FJ5002: A Potent Telomerase Inhibitor Identified by Exploiting the Disease-oriented Screening Program with COMPARE Analysis

Imad Naasani, Hideyuki Seimiya, Takao Yamori, and Takashi Tsuruo
Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Kami-Ikebukuro, Toshima-ku, Tokyo 170-8455 [I. N., H. S., T. Y., T. T.], and Institute of Molecular and Cellular Biosciences, University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113-0032 [I. N., T. T.]; Japan

ABSTRACT

To facilitate the search for candidate telomerase inhibitors, we exploited the database of the disease-oriented screening program (DOS) available in our facility by using COMPARE analysis. In primary and arbitrary screening, we were able to identify the alkaloid berberine as a moderate inhibitor with 50% inhibition at ~35 μM. Using this alkaloid as a seed compound in COMPARE resulted in the identification of other berberine-like compounds and mitochondria-accumulating agents as highly related to berberine. Among these compounds, MKT077, a rhodacyanine derivative currently under Phase I clinical trials, showed a potent inhibitory effect with 50% inhibition at ~35 μM. Using MKT077 as an upgraded seed for a new round of COMPARE analysis, we identified rhodacyanine FJ5002, a close derivative of MKT077, as the most potent telomerase inhibitor with 50% inhibition at ~2 μM. Long-term cultivation of U937, a human leukemia cell line, with subacute concentrations of FJ5002 resulted in population-doubling-dependent changes characterized by progressive telomere erosion (from ~10 to ~4.0 kb), increased chromosome abnormalities, and senescence/crisis-like features. These results indicated that FJ5002 is a genuine and effective antitelomerase agent.

INTRODUCTION

Cancer is a chaotic set of disorders, and each type presents a unique problem with respect to treatment. In the hope of combating the heterogeneous nature of cancer and to identify the most effective compounds against each type of cancer, screening programs such as the Developmental Therapeutics Program (DTP) of the National Cancer Institute (1, 2) and the DOS3 of our foundation (3) were established. The concept of these programs is based on correlating the in vitro performance of tested compounds against a panel of diverse human cancer cell lines (growth inhibitory patterns or “fingerprints”) with performance against the corresponding clinical cancers. Apart from the initial goal of these screening programs, exploiting the collected data by informatic approaches seems to be effective in extracting rich and fundamental information on novel pharmacological modes, biological differences among cancers, and completely new avenues in drug discovery (4). By using COMPARE analysis, a bioinformatic approach of categorizing antitumor agents according to their growth-inhibitory patterns against different human cancer cell lines, we report here a novel strategy for dissecting various agents showing a strong and relatively specific telomerase-inhibitory activity. We were able to identify the alkaloid berberine as a potent telomerase inhibitor identified here, which was effective in inducing telomere erosion and cellular senescence when tested on proliferating U937 human leukemia cells in culture dishes. This compound seems to be a suitable candidate for additional in vivo studies aimed at using telomerase as a novel target for chemoprevention and chemotherapy.

MATERIALS AND METHODS

Reagents and Cell Lines. FJ5002 and other rhodacyanines were kindly provided by Fuji Photo Film Co. (Kanagawa, Japan). Berberine chloride was purchased from Sigma Chemical Co. (St. Louis, MO). Oligonucleotide primers were synthesized and purified by Sawady Technology (Tokyo, Japan). Characterization of 38 cell lines of the DOS was described previously (3). All of the cell lines were grown on RPMI 1640 (Nissui, Co., Tokyo) supplemented with 100 μg/ml kanamycin and 5% fetal bovine serum. Chemosensitivity test in the DOS was performed using a 48-h assay with sulforhodamine B, as described previously (15). The main measure of chemosensitivity used in the database and calculations of correlation factors was GI50. For prolonged passage experiments, U937 human leukemia cells that were derived from a single stock culture were maintained in RPMI 1640 supplemented with 100 μg/ml kanamycin and 10% fetal bovine serum and were passaged as four lineages at a density of 5 × 10^4 cells/ml medium in 10-cm dishes, counted (Coulter), and passaged every three days (at a split ratio of 1:10) with the addition of fresh drug (aqueous solution) at the indicated concentration at each time. Control lineages were handled exactly as the treated lineages. Subcultures were maintained in parallel as four separate passage lineages. However, lineages at crisis were replaced by proliferative ones at the same population doubling to maintain the total number of subcultures at four. Adult NHLF cells were purchased from SANKO (Tokyo, Japan) and maintained according to the protocol provided by the supplier; they were passaged in separate lineages exactly as described for U937 cells.

Detection of Telomerase Activity in Cell Lysates. TRAP assay was performed as described previously (8, 16) with the addition of an internal competitive standard, ITAS (17) and a modified CX primer, CXext (18). Buffered solutions of test compounds were added (5 μl each) to 45 μl of telomerase reaction buffer. Telomerase products were resolved by PAGE and visualized by staining with SYBER Green (Takara, Tokyo). For quantitative measurements in Dixon analysis, the reaction conditions (e.g., primers’ concentrations, PCR cycle number) were optimized in preliminary experiments to be in the dynamic range with the correlation coefficient (r) between the signal (log) and the equivalent number of cells in the extract (log) exceeding 0.99. In all of the assays, a cell lysate equivalent to 10^6 cells was used in each reaction. Calculations were carried out by densitometric analysis using the NIH Image software version 1.60.

Chromosome Analysis in Metaphase Cells. Cells were incubated in 0.1 μg/ml colcemid for 1 h, harvested, incubated for 8 min at 37°C in 0.075 M KCl, and then fixed in freshly prepared methanol/acidic acid (3:1 vol/vol) solution. Cells were preserved at -20°C and, when needed, dropped onto cleaned slides and oven-dried at 50°C overnight before use.

Q-banding. Slides prepared as above were incubated in 50 μg/ml quinacrine mustard in MacIlvaine solution (pH 5.6) for 7 min at room temperature,
briefly washed with tap water, and incubated again in MacIvaine buffer for 7 min. Each slide was carefully wiped, supplemented with a 20 μl of an antifade solution (SlowFade, Molecular Probes, Oregon) per slide, covered with a coverslip (0), and sealed. One hundred metaphases from each treatment were examined for quantification of chromosome abnormalities.

**PRINS Analysis.** PRINS labeling of telomeres (19) was performed according to the instructions provided by the supplier (Boehringer Mannheim, Mannheim, Germany) with minor modifications. Briefly, by using the heating block of a thermal cycler (model 9600, Perkin-Elmer, Norwalk, CT), the slides were heated at 96°C for one min; then, 28 μl of a reaction mixture containing the telomere-specific probe, Taq polymerase, and digoxigenin-labeled deoxyribonucleotides were added. Each slide was quickly covered with a coverslip and heated for another 4 min at 96°C. The temperature was then regulated down to 62°C and left for 30 min to allow for progress of polymerization reaction. Slides were then treated with a stop buffer (50 mM EDTA, 50 mM NaCl (pH 8.0)), followed by a treatment with FITC-labeled antidigoxigenin antibodies. The slides were then washed and counterstained with 20 μl of propidium iodide (0.03 μg/ml) per slide in an antifade solution (SlowFade), covered with a coverslip (0), and sealed. Twenty-five spreads of each treatment were examined, and the telomeric FITC signals were quantified on equal backgrounds (see “Microscopy” below).

**Microscopy.** A Zeiss Axioshot2 fluorescence microscope equipped with a KAF1400-G2 CCD camera (Photometrics) was used. Images visualized with a ×60 objective were captured and saved into a Macintosh computer in a PICT format and then noise-filtered, corrected for background, and merged using Adobe Photoshop (Adobe Systems, Inc., San Jose, CA) files.

**Telomere Length Analysis.** Telomere length was determined by analyzing the mean length of the TRFs. The genomic DNA was extracted from pooled parallel subcultures [to avoid possible selection due to the wide divergence of telomere lengths (20)] by an anion exchange column method (QIAGEN). Approximately 4 μg of DNA were digested by HindIII/RsaI and processed for Southern blotting and chemiluminescence detection according to the instructions provided by the manufacturer of the TeloQuant assay kit (PharMingen, San Diego, CA). Films were quantified using an ATTO densitometer/software system.

**Flow Cytometry.** DNA contents and cell cycle phases were analyzed as described previously (16) using a fluorescence-activated cell sorter (FACScan, Becton Dickinson, San Jose, CA).

**HPLC.** FJ5002 was extracted from treated cells by ethanol (70%) and sonication (extraction ratio was 100 μl solvent:1 × 10⁶ cells), and the supernatant (100 μl) was injected into an HPLC system (Shimadzu LC-6A) equipped with an UV detector (Shimadzu SPD-6AV) set at 335 nm and with an Intersil ODS-2 reverse-phase column. The mobile phase consisted of 30% methanol:triethanolamine:acetic acid [30:70:0.2:0.2 (pH 7.0)].

**Determination of Melting Temperature.** Denaturation profiles were obtained for the telomeric sequence d₅'-AGGGTTAGGGTTAGGGTTAGGG3'- (10 μM; Ref. 21) in the presence or absence of FJ5002 in 10 mM sodium cacodylate and 100 mM NaCl buffer (pH 7.0). Solutions were heated for 5 min at 90°C and then slowly cooled to room temperature over 30 min. Solutions were then placed into a quartz cuvette and heated from 20°C to 84°C with absorbance at 295 nm recorded over a 5-min equilibration time at increments of 0.6°C identified MKT077, a rhodacyanine that is currently under Phase I/II clinical trials (22), as a potent telomerase inhibitor with 50% inhibition at ~2 μM (Table 2; Fig. 2, b and d; Fig. 3a). Using this compound as a new seed for a third round of COMPARE yielded a repeated list of related compounds all of which had already appeared in the first and second rounds, indicating that FJ5002 was the strongest inhibitor that could be detected within the capacity of the available database. It is clear from Fig. 3a that a more than 17-fold increase in inhibition potency could be achieved through the use of COMPARE analysis.

In the next step, we assessed the inhibition kinetics of FJ5002 by Dixon plot analysis at two different concentrations of the TS primer (8) below saturation. Telomerase inhibition by FJ5002 seemed to be substrate-dependent and competitive with an inhibition chloride, an alkaloid found in many plants, as a moderate inhibitor with 50% inhibition at ~35 μM (Fig. 1, a and b; Fig. 3a).

**MKT077 Is a Potent Telomerase Inhibitor.** When berberine was used as an initial seed in COMPARE, other berberine derivatives and mitochondria-accumulating agents were found to be the most distinguishable in the resultant list of berberine-related compounds (Table 1). Testing the telomerase inhibitory effect of the top 20 related agents (r > 0.6) identified MKT077, a rhodacyanine that is currently under clinical trials (22), as a potent telomerase inhibitor with 50% inhibition at ~2 μM (Fig. 2, a and c; Fig. 3a).

**FJ5002 Is the Most Potent Telomerase Inhibitor.** Another round of COMPARE, but this time with the upgraded seed MKT077, resulted in the identification of the rhodacyanine FJ5002 as a more potent telomerase inhibitor with 50% inhibition at ~2 μM (Table 2; Fig. 2, b and d; Fig. 3a). Using this compound as a new seed for a third round of COMPARE yielded a repeated list of related compounds all of which had already appeared in the first and second rounds, indicating that FJ5002 was the strongest inhibitor that could be detected within the capacity of the available database. It is clear from Fig. 3a that a more than 17-fold increase in inhibition potency could be achieved through the use of COMPARE analysis.

**RESULTS**

**Berberine Is a Moderate Inhibitor for Telomerase.** In the case of a telomerase inhibitor, the proper seed for COMPARE should be any compound that is included in the DOS database and has at least minimal inhibitory effects against telomerase. To identify such a seed compound, we launched a random screening with compounds of diverse structures and properties. There was no particular reason for selecting any compound; however, compounds known to inhibit Taq polymerase in the TRAP assay (i.e., nucleosides) were avoided. After testing about 30 different molecules using the TRAP assay with cellular lysates from a representative human cancer cell line (U937 monoblastoid leukemia cells), we were able to identify berberine
G-quartet structure as a driving force (23, 24). Changes in G-quartet melting temperature of the telomeric sequence d5’-AGGGTTAGGGTTAGGGTTAGGG3’ in the presence of FJ5002 were monitored using the method of Mergny et al. (21). As shown in Fig. 3c, FJ5002 had no effect on the G-quartet structure at a molecular stoichiometry (substrate/inhibitor) that was similar to the one used for elaborating the inhibitory effect by the TRAP assay. This finding is in agreement with a model in which FJ5002 inhibits telomerase by a direct interaction. The fact that FJ5002 did not inhibit Taq polymerase in the TRAP assay was by itself evidence for its relative specificity against telomerase.

### Long-Term Cultivation of Cancer Cells in the Presence of FJ5002.

To examine the long-term effect of FJ5002 on U937 cells, it was necessary to identify the drug concentration window in which telomerase could be inhibited but without extensive inhibition of cell proliferation. On the basis of a series of preliminary experiments and HPLC analyses, we found that FJ5002 could accumulate in U937 cells at an intracellular concentration of ~1000 nM (~20-fold) when used at an extracellular concentration of 50 nM. This level was approximately one-half of the maximal level (~2000 nM) that exhibited cytotoxicity within four to five population doublings. Accordingly, we used 50 nM as the treatment concentration in the long-term cultivation experiments that followed.

During long-term cultivation, cells were monitored by microscopic and flow cytometric analyses and by telomere-length estimation using Southern blotting and PRINS. Within the first 20–30 days of passage, no noticeable morphological differences could be detected between treated and untreated control cells except that the growth rate of treated cells was about 20% slower than that of the control cells (Fig. 4a). However, from about day 40 and thereafter, treated cells started to exhibit replicative senescence and/or crisis properties, and a marked increase in cells with enlarged size, cloudy cytoplasm, and ragged...
plasma membrane was observed. Furthermore, the cell population with positive SA-\(\beta\)-Gal, a senescence marker (25), increased from 10\% at about day 4 to 70\% at about day 90 (Fig. 4c). Although an annexin-binding test of apoptosis showed a 20\% increase in the apoptosis rate of the cells at crisis, flow cytometry and DNA fragmentation tests failed to detect a clear pattern of apoptosis in these cells (data not shown). It seems that the cell death at crisis was mediated by both apoptic and nonapoptic pathways. The crisis state observed here closely resembled the M2 crisis state in which the cells show a continuing mitotic activity, a positive staining with SA-\(\beta\)-Gal, and a coexisting rate of cell death (26).

We noticed, however, that, in the lineages of cultivation (day 100) that followed, the cells started to gradually acquire a tendency to recover from the senescence/crisis events and regain viability. To investigate the mechanism of the recovery process, we examined the level of FJ5002 in the recovering lineages and found that the accumulation level of FJ5002 was markedly compromised to one-third of that observed in the original lineage [probably because of multidrug resistance activation inasmuch as the recovering cells also showed cross-resistance to vincristine (data not shown)]. On the basis of this finding, we increased the treatment concentration to achieve the initial accumulation levels (500 to approximately 1000 nM) and closely monitored and adjusted FJ5002 levels in the lineages that followed. This resulted in the reappearance of senescence/crisis events after a few passages.

Fig. 5 shows telomere erosion as determined by estimation of the length of TRF in U937 and NHLF cells.
(0.25 μM for 96 h, three rounds of cell division), FJ5002 did not induce any detectable loss in telomeres (Fig. 5a), indicating that this drug did not actively degrade the telomeric ends by causing DNA damage through intercalation or cross-reaction, a mechanism that was recently observed with cisplatin (27). Interestingly, telomere erosion was concentration-responsive, and showed dynamics that were similar to the cell viability dynamics throughout the course of cultivation (Fig. 5b, c, e). Flow cytometric analysis of the changes in cell cycle phases revealed a gradual increase in the aneuploidy fraction (Fig. 6a and b). This was further confirmed on an individual cell basis using microscopic examination of chromosomal spreads from late passages by Q-banding for general chromosomal abnormality quantification, and by PRINS technique for direct individual telomere length visualization (Fig. 6c and d). The frequency of chromosomal abnormalities (such as end-to-end association, circularization, and translocation) markedly increased in treated cells (abnormality rate was 0.4 and 0.8 event/spread in control and treated cells, respectively, at day >116). On the other hand, the telomeric signal in PRINS analysis of treated cells was, in general, weak, and the number of chromosome ends without FITC signal was 3-fold higher than that of control cells at the same passage day (the rate was 1.3 and 3.7 deleted spot/spread in control and treated cells, respectively, at day 153).

DISCUSSION

The chaos theory is emerging as the new form of analysis for studying complex biological systems such as cancer (28). The plethora of chemotherapeutic agents available today with diverse pharmacological properties is conceived as a chaotic existence that if managed properly, it would be more efficient in meeting with the chaotic nature of cancer. Informatic approaches like COMPARE, provide a powerful mean for managing, de-convoluting and matching the two patterns of chaos, i.e., cancer and chemicals.

Our study demonstrated a novel application of the above concept by exploiting the DOS database to define inhibitors for newly suggested targets (like telomerase) in cancer therapy. Our cell panel of 38 different and well-characterized human cancer cell lines from 10 different tissues (lung, ovary, CNS, blood, stomach, colon, kidney, skin, breast and prostate) is more than sufficient for reflecting the chaotic nature of cancer. Similarly, our DOS database, despite being young, contains more than one thousand standard and experimental anticancer agents, and thus, it is likely to cover almost all of the known and unknown pharmacological trends in cancer.

When we tested various chemical modifications on berberine (palmatine, dehydroberberine, 13-propylberberine, 13-methyldehydroberberine, changing the methoxy with a hydroxy or ethoxy groups), none of these modifications provided a satisfactory telomerase inhibitory effect (data not shown). This indicates that the strategy of using DOS and COMPARE is very helpful in facilitating the tedious and laborious screening for effective inhibitors. After the discovery of berberine, a few molecules (~20 new molecules) were screened to obtain a 17-fold stronger inhibition. Telomerase inhibitory activity did not seem to be related to the property of accumulation in the mitochondria...
because rhodamine123, a typical mitochondrial accumulating agent, was almost ineffective in inhibiting telomerase (Fig. 2, a and c).

Although the growth response of the cell line panel in the 48-h drug-test period does not reflect the toxicity buildup at a lineage level, a property that is expected in the case of specific telomerase inhibitors, the strategy of screening presented here is still effective because it identifies molecules with intrinsic similarity of interaction with the same spectrum of the chaotic biomolecular divergence in cancer cells. The final inhibitor, FJ5002, was tested on live cancer cells at a proliferation-permissive concentration and found to be effective in inducing telomere erosion and loss of cellular viability. Because in vitro (cell-free) telomerase activity was nearly equal in lysates from treated and untreated cells before entering crisis (data not shown), FJ5002 seems to have a reversible effect, and telomerase can regain activity after an approximate 100-fold dilution during the detergent extraction step of TRAP assay. Although FJ5002 at higher concentrations (0.25–5 μM for 96 h) induced a dramatic growth arrest with marked telomerase inhibition (data not shown), the affected cells did not show any features characteristic of replicative senescence or crisis (e.g., SA-β-Gal positivity, telomere shortening, increased cell size). This indicates that the loss of cellular viability observed in the prolonged passage experiment was due to a slow buildup of a heritable defect, which is most likely the gradual telomere erosion during cell division. After the adjustment of FJ5002 intracellular concentration to 500–1,000 nM, the average telomere lengths showed a steep shortening at a rate of ~100 bp per division, consistent with the speculated speed of telomere erosion in the absence of telomerase (because of the DNA end replication problem). That the onset of telomere erosion coincided with a minimal range of intracellular concentrations of FJ5002 has a significant implication on the pharmacodynamics of targeting telomerase in cancer therapy. These findings suggest that the possible future clinical application of telomerase inhibitors should be accompanied by cautious monitoring of the minimal therapeutic dose to ensure persistent telomerase inhibition; otherwise, telomeres would regain their original lengths.

As a negative control, we examined the prolonged passage of adult NHLF cells (which have no telomerase activity) in the presence of 50 nM of FJ5002 and could not find any detectable difference between treated and untreated cells in terms of the rate of telomere erosion (Fig. 5d) or morphology (data not shown). In addition to the results of telomerase inhibition in a cell-free system, the population doubling-dependent telomere erosion and crisis of U937 cells, failure of FJ5002...
to induce similar effects in telomerase-negative NHLF cells, and inability of FJ5002 to interact directly with telomeric DNA (Fig. 3c, and data not shown), altogether suggest that FJ5002-induced telomere shortening is mediated by a telomerase-inhibitory mechanism. It is noteworthy that the TRF values shown in Fig. 5e represent the average of the total lengths of all of the telomeric and subtelomeric regions. The actual telomere shortening observed here could reach far more critical levels (>1 kb), particularly if we considered a cutoff value at the lower part of the TRF lane in Southern blotting. This is in agreement with previous findings that showed that transformed human cells enter crisis once TRF reaches a length of >4 kb (14). Strahl and Blackburn (29) have shown that some nucleoside analogues (dideoxyguanosine and azidothymidine at >10 and >100 μM, respectively) inhibit telomerase and induce telomere shortening in some T- and B-cell cultures but without detectable loss of cell viability. Although their study lacked in-depth investigations on inhibition mechanisms and cellular viability, they attributed the telomere-shortening resistance of these cells to the presence of telomerase-independent mechanisms for chromosome healing. In the present study, there was also a remaining subpopulation of U937 cells that continued to proliferate despite the critical level of telomere shortening. In these cells, telomerase remained sensitive to FJ5002 in vitro (data not shown). Recently, two mechanisms were proposed for explaining the survival of telomerase-deleted fission yeast, one by chromosome circulization and the other presumably by recombination (30). We are presently investigating these two possibilities for explaining the survival mechanism of the final lineages of U937 leukemia cells observed in the present study. Given that U937 lymphoblastoid cells, similar to cells in the immune system, are equipped with advanced DNA recombination systems for receptors and antibody production, the possible existence of a recombination mechanism for survival is highly probable. Like many other cancer cells, U937 cells are heterozygously mutant for the tumor suppressor gene p53 (data not shown); thus, they could survive for a long period with short telomeres because p53 (and possibly other suppressors) senses the early events of telomere erosion as an irreparable DNA damage and induces growth-arrest before reaching a marked shortening of telomeres (31). It would also be interesting to test the long-term effect of FJ5002 on p53-positive cell lines.

In conclusion, our novel application of COMPARE informatics for exploiting the database generated from our cell-panel screening program enabled the identification of FJ5002 as a potent telomerase inhibitor. This compound could be potentially useful as a lead molecule for additional in vivo and clinical studies aimed at telomerase targeting.

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