Mechanism of Human Telomerase Inhibition by BIBR1532, a Synthetic, Non-nucleosidic Drug Candidate*

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Telomerase, a ribonucleoprotein acting as a reverse transcriptase, has been identified as a target for cancer drug discovery. The synthetic, non-nucleosidic compound, BIBR1532, is a potent and selective telomerase inhibitor capable of inducing senescence in human cancer cells (1). In the present study, the mode of drug action was characterized. BIBR1532 inhibits the native and recombinant human telomerase, comprising the human telomerase reverse transcriptase and human telomerase RNA components, with similar potency primarily by interfering with the processivity of the enzyme. Enzyme-kinetic experiments show that BIBR1532 is a mixed-type non-competitive inhibitor and suggest a drug binding site distinct from the sites for deoxyribonucleotides and the DNA primer, respectively. Thus, BIBR1532 defines a novel class of telomerase inhibitor with mechanistic similarities to non-nucleosidic inhibitors of HIV1 reverse transcriptase.

The reactivation of telomerase is a key requisite for human cancer cells to attain an unlimited proliferation potential and is regarded as an essential alteration in the physiology of the tumor cell to acquire malignant growth. (2–5). The underlying concept, namely telomere maintenance by telomerase, has been demonstrated for 85–90% of human cancer specimens from a large range of different cancer types (6). Constitutive overexpression of the enzyme in various presenescent and normal cells conveyed an unlimited growth potential onto these cells (3), confirming further the role of telomerase in the immortalization process. In contrast, inhibition of telomerase results in telomere-shortening, subsequent growth arrest, and senescence in a wide range of tumor cell lines. This has been demonstrated by expressing a dominant-negative form of telomerase in immortal tumor cell lines (7, 8) and, pharmacologically, by the use of the small molecule telomerase inhibitor, BIBR1532 (1). These data underscore that telomerase may represent a valuable target for novel anticancer therapies.

Telomerase is a ribonucleoprotein that acts as a reverse transcriptase (RT)1 by using a small region of its RNA subunit, hTR, as a template for the synthesis of telomeric DNA (9–11). Reverse transcription itself is catalyzed by the telomerase protein subunit, hTERT. Since catalytically active telomerase has been assembled from recombinant hTERT protein and in vitro transcribed hTR (12, 13), these subunits are regarded as the telomerase core enzyme. In vivo, however, human telomerase exists as a high molecular weight complex with an estimated molecular mass of 1000 KDa (14–17). This large size may be due to the multimeric nature of human telomerase and the association of the telomerase core components, hTERT and hTR, with several telomerase-associated proteins. These diverse proteins may play an important role in telomerase biology, regulation and stability, or may modulate the interaction with telomeres in vivo; however, they are not considered to exert a direct function in catalysis (12, 13, 18).

In vitro telomerase is able to elongate a short single-stranded DNA in a processive manner by adding multiple TTAGGG repeats to the 3′-end of a suitable DNA primer. Since the enzyme appears to pause after synthesis of each set of six nucleotides representing a single telomeric repeat, a typical pattern of product bands spaced at six-nucleotide intervals is observed. Once the 5′ boundary of the template is copied the DNA substrate is thought to either translocate during processive synthesis, or it may dissociate from the enzyme. Thus, to allow addition of multiple telomeric repeats, translocation and re-initiation must take place after each cycle of template copying. The mechanisms involved are not elucidated yet, but a critical factor could be the dimeric nature of human telomerase with two hTERT and two hTR molecules present per functional telomerase complex (19, 20).

Because of the structural and mechanistic similarity between hTERT and reverse transcriptases, it has been hypothesized that known reverse transcriptase inhibitors may potently inhibit human telomerase. HIV1-RT has been successfully inhibited by nucleoside analogs, which bind to the dNTP binding site (21) and by non-nucleoside inhibitors (NNRTI), which bind to a hydrophobic pocket near the catalytic center resulting in a distortion of the active site (22–24). However, all NNRTI and nucleoside analog inhibitors of HIV1-RT tested were found to be inactive or to exhibit only weak inhibitory activity toward human telomerase (25–27), suggesting

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1 The abbreviations used are: RT, reverse transcriptase; hTERT, human telomerase reverse transcriptase; hTR, human telomerase RNA; HIV, human immunodeficiency virus; BIBR1532, 2-((E)-3-naphthalen-2-yl-but-2-enoylamino)-benzoic acid; TRAP, telomeric repeat amplification protocol.
that structural differences between these two families of reverse transcriptases are sufficient to allow specificity of the inhibitors. Additional strategies for inhibition of telomerase have been explored, including antisense approaches directed against hTR (28, 29), compounds targeting telomeric DNA (30, 31), and small molecule drugs (32).

In the present study, we present the initial characterization of the mode of telomerase inhibition by BIBR1532, a synthetic small molecule inhibitor of human telomerase (1).

**MATERIALS AND METHODS**

Deoxyribonucleotides were from Amersham Biosciences. 1,4-Dithiothreitol was from Roche Diagnostics, and phenylmethylsulfonyl fluoride was from Invitrogen. \([\alpha^32P]dCTP (1 \text{ mCi}/100 \mu\text{l})\) was purchased from Hartmann Analytic. The PCR primer forward (tea-fw: 5'-CAT ACT GGC GAC AGT T-3') and reverse (tea-rev: 5'-GCC GCG CCC TTA CCC TTA CCC TTA CAA-3') were from Carl Roth GmbH.

**Preparation of Telomerase-enriched Extracts**—Crude HeLa nuclear extracts (Computer Cell Culture Center, Seneffe, Belgium) were enriched for telomerase activity with a one-step chromatography on Q-Sepharose column (HiTrap Q HP, Amersham Biosciences). The buffer used was 20 mM Tris-Cl (pH 8.0), 100 mM EGTA, 100 mM EDTA, 1 mM MgCl₂, 10% (w/v) glycerol, complemented with different concentrations of KCl (BCE100, 100 mM; BCE250, 250 mM; BCE500, 500 mM; BCE1000, 1 M). The 1-ml column was equilibrated in BCE100 buffer. The following steps were carried at 4°C. 2 mg of HeLa nuclear extract was diluted in a large volume of BCE100 and loaded twice on the column at 0.5 ml/min. The column was washed at 0.5 ml/min with 4 volumes of BCE100 and loaded twice on the column at 0.5 ml/min. The column was washed at 0.5 ml/min with 4 volumes of BCE100 and 3 volumes of BCE250. Most of the proteins were eluted by washing with 4 volumes of BCE500. Telomerase activity was collected as 1-ml fractions by elution with 10 volumes of BCE1000.

**FIG. 1.** BIBR1532 inhibits preferentially the processivity of human telomerase. The inhibition of human telomerase partially purified from HeLa nuclear extract was tested in a direct telomerase assay (A) or a PCR-based TRAP assay (B) as described under “Material and Methods.” The arrows indicate the product bands used for quantification. C, the intensities of TRAP products referred to as band 1 (closed circles), band 5 (open circles), band 7 (filled triangles), band 9 (open triangles), band 11 (filled squares), and band 15 (open squares) were normalized to the intensities of the corresponding products in the control without inhibitor and plotted against the concentration of BIBR1532.
BIBR1532 inhibits recombinant telomerase.

A. direct telomerase assay. Telomerase was reconstituted with insect cell expressed hTERT and in vitro transcribed hTR. Recombinant His-tagged hTERT associated with hTR was purified as described previously via specific affinity chromatography with an oligonucleotide directed against the TR template sequence (19). The final concentrations in the reaction mixture were 25 °C for 3 h and stopped by addition of 50 µl of RNase mix (0.1 mg/ml RNaseA, Roche Applied Science)-100 µl RNaseT1 (Roche Applied Science) in 10 mM Tris-Cl (pH 8.3) and 20 mM EDTA

BIBR1532

B. TRAP assay—The TRAP was performed as described previously (17, 33). In this assay BIBR1532 has no inhibitory effect when added after the telomerase reaction. Unless indicated, the dNTPs were each present at 80 µM and the oligonucleotide primer at 700 nm. The telomerase fraction and BIBR1532 were preincubated for 15 min on ice in reaction buffer. After addition of different concentrations of the variable reactant, the reaction was initiated by incubation at 23 °C for 3–6 min, stopped by heating at 90 °C for 90 s, and kept on ice. Before proceeding with the PCR reaction, the variable reactant was adjusted to a final concentration of 50 µM for each dNTP and 700 µM for the DNA primer. The PCR mix was added, and PCR was performed for 27 cycles (30 min at 94 °C, 30 min at 50 °C, 90 min at 72 °C). The amount of radiolabeled products was either quantified by liquid scintillation counting after precipitation with 5% trichloroacetic acid or on a PhosphoImager after fractionation on a 6% acrylamide/bisacrylamide gel.

Determination of Kinetic Constants—To determine the kinetic constants of BIBR1532 inhibition, TRAP assays were carried out in triplicate in the presence of varying concentrations of substrate and inhibitor. Reactions were performed at linear conditions, and the results of three assays were used for further calculations. The reaction products were precipitated with 5% trichloroacetic acid, collected on glass fiber filters (Millipore), and the amount of incorporated radioactivity was determined by liquid scintillation counting. For velocity plots, the incorporation of radioactivity (in counts/min) was blotted against the variable substrate. $K_v$ values were calculated as the concentration of variable substrate required to reach $V_{max}$. The inhibition constants were determined from secondary plots (Lineweaver and Burk). $K_i$ and $aK_i$ correspond to the $x$-intercept of the linear replots slope = $f(I)$ and $y$-intercept = $f(I)$, respectively.

BIBR1532 Is an Inhibitor of Cellular and Recombinant Telomerase—BIBR1532 has been identified as a potent and selective inhibitor of human telomerase (1). To obtain a better understanding of the mechanism of action exerted by this compound, the mode of telomerase inhibition was addressed in further detail using both native enzyme enriched from HeLa cell nuclear extract as well as recombinant enzyme reconstituted from recombinant hTERT and in vitro transcribed hTR. The effect of BIBR1532 on telomerase activity was analyzed using two published assay methods, a conventional assay (11) relying on a direct measurement of enzyme activity and the PCR-based TRAP assay (33), which includes an amplification step. A shown in Fig. 1A, the native enzyme synthesized long extension products in the conventional assay (lane 1). Increasing concentrations of BIBR1532 inhibit this process in a dose-dependent manner (lanes 2–5). Calculation of the total signal intensity by PhosphoImager analysis revealed an IC$_{50}$ value of $-100$ nm. Noticeably, at low concentrations of the inhibitor, the synthesis of long extension products appears to be more affected than the synthesis of shorter products (lanes 1–3). Inhibition of telomerase activity is also observed in a TRAP assay (Fig. 1B, lanes 1–7). Consistent with the result of the conventional primer extension assay, the synthesis of longer products is preferentially inhibited at low concentrations of BIBR1532 (lanes 2–7). To allow a quantification, the intensity of individual extension products was analyzed by PhosphoImaging, and
dose-response curves were generated for each product. As shown in Fig. 1C, the IC50 value for the shortest product (band 1), which corresponds to the two first cycles of template copying, is \( \sim 750 \text{nM} \). The formation of the two longest products analyzed (bands 11 and 15) is inhibited with IC50 values of 150 nM and 100 nM, respectively.

To determine whether BIBR1532 would directly act on the telomerase hTERT-hTR core enzyme, active telomerase was reconstituted by incubating insect cell lysate containing recombinant hTERT with in vitro transcribed hTR. Reconstituted telomerase ribonucleoproteins were purified by RNA affinity chromatography, and the eluted enzyme was tested in the conventional primer extension assay. As shown in Fig. 2A, the purified reconstituted enzyme catalyzed the formation of the specific hexanucleotide repeat ladder characteristic for human telomerase (lane 1) with the most prominent product bands corresponding to the first four cycles of template copying. Also this reconstituted telomerase was inhibited by BIBR1532 (lanes 4–9). As seen for the native enzyme, the formation of longer products is affected stronger than the formation of the shorter ones (Fig. 2B). The bottom band, which corresponds to the first cycle of template copying (+4), is only weakly inhibited at concentrations of BIBR1532 below 1 \( \mu \text{M} \), whereas the formation of the +16 and +22 products, corresponding to three and four cycles of template copying, respectively, is inhibited with IC50 values of \(-100 \text{nM}\). Interestingly, even in the presence of high concentrations of BIBR1532, no chain termination events (e.g., the appearance of new intermediate products) were observed. Thus, BIBR1532 does not inhibit the catalytic steps during a single round of template copying.

**FIG. 3.** BIBR1532 is a non-competitive inhibitor for the binding of the DNA primer. A, kinetic analysis of human telomerase in a TRAP assay. Telomerase reactions were performed with different quantities of partially purified telomerase as described under “Materials and Methods:” 4.5 ng (open circles), 9 ng (filled circles), 18 ng (open triangles), 45 ng (filled triangles), or 90 ng (filled squares) of total protein. Reactions were stopped after various time points, and products were analyzed as described. Telomerase activity (in counts/min) was plotted against the reaction time. B, direct plot of telomerase activity (18 ng of protein, 6-min reaction time) versus the concentration of DNA primer (nM) in the absence or presence of different concentrations of BIBR1532; no BIBR1532 (open circles), 200 nM (filled circles), 500 nM (filled triangles), or 1 \( \mu \text{M} \) BIBR1532 (filled diamonds). C, Michaelis-Menten constants of the DNA primer in the absence (\( K_m \)) or in the presence (\( aK_m \)) of 1 \( \mu \text{M} \) BIBR1532. D, affinity constants of BIBR1532 in the absence (\( K_i \)) or in the presence (\( aK_i \)) of DNA primer.

**BIBR1532 Is a Mixed-type Non-competitive Inhibitor of Human Telomerase**—To characterize the mode of inhibition by BIBR1532 as a function of the four substrates required for telomerase activity in vitro, namely, dATP, TTP, dGTP, and a DNA primer, a series of enzyme kinetic experiments were performed. The conditions for the linear phase of primer elongation were determined in an initial experiment in the presence of saturating substrate concentrations and variable amounts of partially purified native telomerase. As shown in Fig. 3A, a linear correlation between reaction time, enzyme concentration, and the generation of telomerase products was observed for incubations below 10 min. Therefore, for the subsequent experiments reaction times between 4 and 6 min were used, and velocity curves were determined for different substrate concentrations in the presence or absence of BIBR1532. As shown in Fig. 3B, a hyperbolic curve was obtained when telomerase activity was plotted as a function of the DNA primer concentration in the absence of the inhibitor (open circles). The maximum enzymatic reaction (\( V_{\text{max}} \)) was reached with primer concentrations above 150 nM. In the presence of increasing amounts of BIBR1532, a clear reduction in \( V_{\text{max}} \) was observed, a feature characteristic for a non-competitive inhibition. At the highest inhibitor concentration tested (1 \( \mu \text{M} \)), \( V_{\text{max}} \) was decreased by 60%. The data were used to calculate the Michaelis-Menten constant of the DNA primer in the absence (\( K_m \)) and in the presence of BIBR1532 (\( aK_m \)) and to calculate the binding constants of the inhibitor in the absence (\( K_i \)) and in the presence (\( aK_i \)) of the DNA primer. As shown in Fig. 3C, the \( K_m \) and \( aK_m \) values for the DNA primer were not significantly different, suggesting that BIBR1532 does not affect the binding of the DNA primer to the enzyme. However, a small, but significant, increase of \( aK_i \) (750 nM) over \( K_i \) (500 nM) was observed (Fig. 3D), indicating a slightly higher affinity of BIBR1532 to the free enzyme than to the enzyme-DNA primer complex.

The velocity curves and the resulting double-reciprocal plots (Lineweaver-Burk diagrams) were determined for each of the three deoxyribonucleotides in the presence or absence of
BIBR1532 (Figs. 4 and 5, A–C, respectively). As shown in Fig. 4, for each of the three dNTPs a pronounced decrease in $V_{\text{max}}$ was observed in the presence of BIBR1532, indicating a non-competitive mode of inhibition for the deoxyribonucleotides also. The Michaelis-Menten constants for each of the three dNTPs increased in the presence of the inhibitor (Fig. 4D). At $1 \mu M$ BIBR1532, the $K_m$ values for dATP (5 $\mu M$), TTP (7 $\mu M$), and dGTP (14 $\mu M$) increased to 11, 14, and 23 $\mu M$, respectively ($\alpha K_m$, Fig. 4D), suggesting for each dNTP a lower affinity to the telomerase-BIBR1532 complex. The affinity constants of BIBR1532 were calculated to be 250–300 nM for the nucleotide-free enzyme ($K_i$, Fig. 5D) and 600–800 nM for a telomerase-dNTP complex ($\alpha K_i$, Fig. 5D). This indicates a tight binding of the drug to the nucleotide-free enzyme and a 2–3-fold lower affinity to each enzyme-dNTP complex. In the corresponding Lineweaver-Burk plots these conditions resulted in the intersection of the control (no inhibitor) and the “plus inhibitor” curves above the $1/[S]$-axis, since $\alpha K_i$ was larger than $K_i$ (Fig. 5, A–C).

**Fig. 4.** BIBR1532 is a mixed-type non-competitive inhibitor for the binding of dNTP. A–C, telomerase reactions were performed for 6 min with 18 ng of partially purified telomerase as described under “Materials and Methods” in the presence of variable concentrations of dGTP (A), TTP (B), dATP (C), and BIBR1532. Open circles, no BIBR1532; filled circles, 200 nM; filled triangles, 500 nM; filled squares, 1 $\mu M$. Telomerase activity (in counts/min) is plotted versus dGTP (A), TTP (B), and dATP (C) for different concentrations of BIBR1532. D, Michaelis-Menten constants of the deoxyribonucleotides in the absence ($K_m$) or in the presence ($\alpha K_m$) of 1 $\mu M$ BIBR1532.

**Fig. 5.** BIBR1532 is a mixed-type non-competitive inhibitor for the binding of dNTP. A–C, double-reciprocal plot (Lineweaver-Burk) of velocity curves shown in Fig. 4. A–C, D, affinity constants of BIBR1532 in the absence ($K_i$) or the presence ($\alpha K_i$) of the variable deoxyribonucleotide (80 $\mu M$).
The concomitant increase in the value for the affinity constant and a decrease in $V_{\text{max}}$ suggests a mixed-type inhibition, with different binding sites for the substrate(s) and the inhibitor, but with strong influence between the binding of each other (34).

**DISCUSSION**

The definition of the precise mechanism of enzyme inhibition, by pharmaceutically relevant small molecule drug candidates, is of considerable interest as shown for the HIV drugs (22, 23).

In this study we show that BIBR1532 targets directly telomerase core components as telomerase reconstituted from hTR and recombinant hTERT is inhibited by BIBR1532 with potencies comparable with the native enzyme derived from tumor cells. In addition, BIBR1532 exhibits a non-competitive mode of inhibition, which is clearly distinct from the inhibition described using nucleosidic compounds or antisense oligonucleotides. Both for the native and the recombinant enzyme our data show that BIBR1532 does not cause chain termination events but rather inhibits the formation of long reaction products. In particular, the inhibitor leads to an overall reduction in the number of added TTAGGG repeats; the periodicity of six nucleotides, however, is conserved. This suggests that BIBR1532 does not block the basic catalytic steps involved in template copying but specifically impairs the elongation of the DNA substrate after its extension to the 5'-end of the template. Thus, BIBR1532 may affect translocation of the enzyme DNA substrate complex or may promote dissociation between DNA substrate and the enzyme upon completion of template copying. As these steps are most likely unique to telomerase, this may explain the high selectivity of the compound.

In a more detailed analysis the specific kinetic parameters of inhibition by BIBR1532 were determined. We detected only a slight inhibition of the binding of the DNA primer in the presence of BIBR1532. However, BIBR1532 reduced more than 2-fold the affinity for dNTPs. Conversely, binding of deoxyribonucleotides decreased the affinity of the enzyme for BIBR1532. This inhibition profile corresponds to a mixed-type non-competitive inhibition, in which the enzyme has different, but functionally interdependent, binding sites for deoxyribonucleotides and BIBR1532. Two hypotheses may explain such an interference. First, the binding of the substrate or the drug induces a conformational change of the enzyme structure interfering with the binding of the other molecule. Second, the binding site of the drug and the binding site of the deoxyribonucleotides are in close proximity or overlap, creating therefore a steric reciprocal interference for the binding efficiency. The biochemical data described above do not support an allosteric inhibition profile, and thus we favor the latter hypothesis.

Compared with telomerase considerable more details on enzyme structure and function are known about HIV1 reverse transcriptase. The three-dimensional structure of this enzyme is often described as a right hand naming the subdomains as fingers, palm, and thumb, with the catalytic center being located within the palm subdomain (23). All three subdomains are important determinants for HIV1-RT processivity. The non-nucleosidic drug nevirapine inhibits preferentially the translocation step of polymerization (22). Co-crystallization experiments revealed that nevirapine binds into a deep hydrophobic pocket between the palm and the base of the thumb (the “primer grip”) close to, but not overlapping with, the DNA binding site (23). Binding of the drug may either induce repopositioning of the catalytic aspartic acids or may prevent conformational changes required to complete the catalytic cycle. Although the primary sequence similarity between telomerase and HIV1 reverse transcriptase is low, key features in structure and basic mechanism of catalysis appear similar. For example, it has been shown that point mutations in amino acids that are conserved between TERTs and retroviral reverse transcriptases reduced or abolished activity in both types of enzymes or had similar effects on processivity (13, 35–39).

Recently, a detailed mutational analysis undertaken to study the processivity of yeast TERT primer grip and thumb subdomain suggested that telomerase, as its retroviral cousin, may contain hydrophobic pockets between the palm and thumb, which also might be available for binding of small molecule inhibitors (39, 40).

Therefore, the apparent similarities in the mechanism of action of nevirapine on HIV1 reverse transcriptase and BIBR1532 on human telomerase together with the structural and mechanistic similarities of both enzymes are intriguing. However, a detailed understanding of the molecular basis of BIBR1532 inhibition will require the crystal structure analysis of the telomerase-inhibitor complex. For HIV1-RT a cooperative inhibitory effect has been observed when nevirapine and nucleosidic analogs were used concomitantly (22). Therefore, it will be of interest to determine whether inhibition of telomerase by BIBR1532 is potentiated when combined with nucleotide analogs in biochemical *in vitro* assays. Such cooperativity would increase interest in the discovery of nucleosidic telomerase inhibitors.

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