Inhibition of telomerase by linear-chain fatty acids: a structural analysis

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In the present study, we have found that mono-unsaturated linear-chain fatty acids in the *cis* configuration with C₁₈ hydrocarbon chains (i.e. oleic acid) strongly inhibited the activity of human telomerase in a cell-free enzymic assay, with an IC₅₀ value of 8.6 μ M. Interestingly, fatty acids with hydrocarbon chain lengths below 16 or above 20 carbons substantially decreased the potency of inhibition of telomerase. Moreover, the *cis*-mono-unsaturated C₁₈ linear-chain fatty acid oleic acid was the strongest inhibitor of all the fatty acids tested. A kinetic study revealed that oleic acid competitively inhibited the activity of telomerase ($K_i = 3.06 \mu\text{M}$) with respect to the telomerase substrate primer.

The energy-minimized three-dimensional structure of the linear-chain fatty acid was calculated and modelled. A molecule width of 11.53–14.26 Å (where 1 Å = 0.1 nm) in the C₁₆ to C₂₀ fatty acid structure was suggested to be important for telomerase inhibition. The three-dimensional structure of the telomerase active site (i.e. the substrate primer-binding site) appears to have a pocket that could bind oleic acid, with the pocket being 8.50 Å long and 12.80 Å wide.

Key words: computer modelling, enzyme inhibitor, oleic acid, RNA-dependent DNA polymerase.

INTRODUCTION

As telomerase is an attractive target for cancer chemotherapy [1,2], the purpose of the present study was to find inhibitors specific to human telomerase and to analyse the molecular mechanism of the inhibition. It is well known that human telomerase activity is not detectable in most somatic cells, but is up-regulated in most human cancer cells [3,4], and the ectopic expression of telomerase in normal human cells is associated with the direct tumorigenic conversion of normal cells with other oncoproteins [5–7]. It has been demonstrated [8,9] that the expression of a telomerase mutant results in the inhibition of telomerase activity, telomere shortening and death of tumour cells. Therefore clarifying the molecular mechanism of the inhibition should aid the creation of an ideal artificial cancer chemotherapy agent.

To date, several classes of compounds have been reported as potent inhibitors of telomerase, including antisense oligonucleotides and peptide nucleic acids [10–12], ribozymes [13], nucleotide analogue reverse transcriptase (RT) inhibitors [14,15], G-quadruplex-interacting compounds [16–21], anti-tumour alkaloids [22], antisense phosphothioate oligonucleotides encapsulated with transfection reagent [23], isothiazolone derivatives [24], rhodacyanine derivatives [25] and natural compounds [26–29]. On the basis of a functional relationship between telomerase and HIV-RT, as RNA-dependent DNA polymerases, rubromycin analogues, a class of quinone antibiotics with spiroketal systems, have also been discovered as potent telomerase inhibitors [29]. However, they were not suitable as anti-cancer agents and therefore the search for new natural anti-telomerase compounds continues.

Aside from telomerase inhibitors, we have screened inhibitors of mammalian DNA polymerases α and β and found that mammalian DNA polymerases α and β are inhibited by linear-chain fatty acids with the following characteristics: a hydrocarbon chain containing 18 or more carbons, with a free carboxyl end and in the *cis* configuration [30–32]. The mode of binding linear-chain fatty acids to DNA polymerase β has been studied extensively using NMR spectroscopy [33] and revealed that fatty acids specifically bound to the proteolytic N-terminal 8 kDa domain fragment of DNA polymerase β at the DNA-binding groove.

In the present study, we describe a number of linear-chain fatty acids that inhibited human telomerase activity in a cell-free assay. These results suggest that the site of inhibition on telomerase is the same as for other similar polymerases and the inhibition site to which the fatty acids binds can be analysed using computer and three-dimensional (3D) structural modelling. Since the lack of tertiary structural information, especially around the substrate-binding groove of telomerase, renders the design of specific inhibitors difficult, the analysis of binding between the linear-chain fatty acids and telomerase may be used to create an ideal artificial cancer chemotherapy agent.

MATERIALS AND METHODS

Fatty acids

The linear-chain fatty acids were named using the nomenclature described by Weete [34] [(A:B Δ C1–C2), where A is the number of carbon atoms; B, the number of double bonds and C1–C2 the position of each double bond from the carboxyl end of the molecule]. Saturated fatty acids, *cis*-unsaturated fatty acids,

Abbreviations used: 3D, three-dimensional; RT, reverse transcriptase; TRAP, telomeric repeat amplification protocol.

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trans-unsaturated fatty acids and derivatives of C₁₈ fatty acid were purchased from Nu-Chek-Prep Inc.

Enzyme assay for human telomerase

The enzymic activity of human telomerase extracted from K-562 cells was performed as described previously [29] based on the telomeric repeat amplification protocol (TRAP) [3]. Briefly, the reaction mixture [20 mM Tris/HCl (pH 8.3), 68 mM KCl, 1 mM EGTA, 0.05 % Tween 20, 0.5 μ M T4 gene 32 protein (Boehringer Mannheim), 8 μ g/ml phage MS2 RNA (Boehringer Mannheim), 100 μ g/ml BSA and K-562 lysate equivalent to 5000 cells] was preincubated for 10 min at 25 °C in the absence or presence of various amounts of test compounds dissolved in DMSO. The telomerase reaction was then initiated by the addition of a 200 μ M each of the four dNTPs and a modified telomerase substrate primer, TS-A (5'-AAAAAAAAAAAAAAAAATCCGTC-GAGCAGAGTT-3'), in a total volume of 50 μ l, and the mixture was subsequently incubated for 30 min at 25 °C. After the reaction had been quenched by the addition of 20 μ l of 0.1 M EDTA (pH 8.0)/20 μ g/ml glycogen, TS-A oligonucleotides elongated by telomerase were extracted sequentially in phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) and chloroform/isoamyl alcohol (24:1, v/v) and then precipitated in ethanol. The precipitated materials were amplified by PCR using 6-FAM-labelled ACX primer (5'-GCGCGGCTTACCCTTACCCTTACCCTAACCC-3') as described by Ueno et al. [29]. The resulting PCR-amplified telomerase product (1 μ l) was mixed with a fluorescent size standard (1 μ l of GS-350 ROX; Applied Biosystems) and separated and monitored by capillary electrophoresis (Genetic Analyzer 310; Applied Biosystems). The amount of the product was integrated and quantified with computer software (GeneScan Analysis 2.1; Applied Biosystems). It should be noted that, although some fatty acids used in the present study inhibited the activity of *Taq* polymerase and thereby strongly suppressed the amplification by PCR, the extraction step prior to PCR allowed the amplification of the telomerase product by PCR to occur without interference from the test compounds [29].

RESULTS AND DISCUSSION

Linear-chain fatty-acid-mediated inhibition of human telomerase

We tested the ability several classes of linear-chain fatty acids to inhibit telomerase-mediated addition of hexameric nucleotide repeats (5'-TTAGGG-3')_n on to the ends of TS-A by using the modified TRAP assay described by Ueno et al. [29]. A series of saturated fatty acids with hydrocarbon chain lengths from 10–24 carbons and at a concentration of 100 μ M were initially tested and showed little effect on telomerase inhibition (Figure 1A), with IC₅₀ values above 500 μ M.

We then investigated the inhibitory effect of unsaturated linear-chain fatty acids in the *cis* configuration with hydrocarbon chains lengths of 12–24 carbons on telomerase activity. As shown in Figure 1(B), 100 μ M C₁₆ fatty acid, such as palmitoleic acid (16:1 Δ^9 *cis*), C₁₈ unsaturated fatty acids and C₂₀ fatty acids, such as icosenoic acid (20:1 Δ^{11} *cis*), significantly inhibited telomerase-mediated addition of the nucleotide repeats, suggesting that an unsaturated hydrocarbon bond in these fatty acids played a critical role in the telomerase inhibition. In particular, oleic acid (18:1 Δ^9 *cis*) was the most potent telomerase inhibitor of the C₁₈ unsaturated fatty acids. In contrast, fatty acids with hydrocarbon chain lengths of either less than 14 or more than 22 carbons at concentrations of 100 μ M were not potent inhibitors of telomerase activity. Also, none of the *trans*-

unsaturated linear-chain fatty acids inhibited telomerase activity (Figure 1C). These results suggest that both the isomeric configuration (*cis* versus *trans*) and the length of the hydrocarbon chain of the fatty acid structure are important in providing a potent telomerase-inhibitory effect.

On the other hand, the inhibition of telomerase activity by linear-chain C₁₈ fatty acids was more potent when the carboxy group was free. When the carboxy group was chemically modified by adding a methyl ester, ethyl ester, acetate or alcohol, or when it was completely removed, these hydrocarbons did not inhibit telomerase activity (Figure 1D). These results show the importance of the free carboxy group in the inhibition.

Figure 2(A) shows the dose–response curves for each of the *cis*-mono-unsaturated linear-chain fatty acids. Among the C₁₄ to C₂₄ mono-unsaturated fatty acids tested, oleic acid (a C₁₈ fatty acid) was the most potent inhibitor of telomerase, with 50 % inhibition occurring at 8.6 μ M. C₁₆ and C₂₀ fatty acids moderately inhibited telomerase, whereas fatty acids with hydrocarbon chain lengths of either less than 14 or more than 22 carbons did not, indicating that the length of hydrocarbon chain is a critical factor in telomerase inhibition. We also examined the effect of the number of *cis*-unsaturated bonds in a fatty acid on the potency of inhibiting telomerase activity using mono-, di-, tri- and tetra-unsaturated C₁₈ fatty acids. As shown in Figure 2(B), a *cis*-mono-unsaturated linear-chain fatty acid (i.e. oleic acid) was again the most potent inhibitor of telomerase among the C₁₈ fatty acids tested. The *cis*-octadecenoic acids have an ethylenic double bond at position 6 (petroselinic acid), 9 (oleic acid) or 11 (vaccenic acid) and the IC₅₀ values for telomerase inhibition were 15, 8.6 and 16 μ M respectively (results not shown). Oleic acid showed the strongest inhibition of these *cis*-octadecenoic acids, suggesting that the position of the double bond was also important for inhibition. *cis*-Di-, tri- and tetra-C₁₈ fatty acids moderately inhibited telomerase activity, whereas a saturated C₁₈ fatty acid (i.e. stearic acid) did not. Therefore one double-bond in the *cis* configuration in the unsaturated linear-chain fatty acids appears to play an important role in the molecular mechanism of the telomerase inhibition.

We have reported previously [31,32] that mono-unsaturated linear-chain fatty acids in the *cis* configuration inhibited the activities of mammalian DNA polymerases α , β and HIV-RT, and that increasing the hydrocarbon chain length of the fatty acids from 18 to 24 carbons led to an increase in the potency of enzyme inhibition. The IC₅₀ values of fatty acids for telomerase inhibition were almost the same as those for the inhibition of DNA polymerases α and β and HIV-RT. We compared the characteristics of the fatty acids that inhibit the activities of telomerase and DNA polymerases, including HIV-RT, and found that the linear-chain fatty acid structures, such as the hydrocarbon chain length, the *cis* configuration of the double bond and free carboxy group, were very similar or almost the same as each other. Therefore the mechanism of inhibition by fatty acids must be similar in both cases.

Mechanism of action of telomerase inhibition by oleic acid

We employed a steady-state kinetic analysis of telomerase inhibition using oleic acid, which showed the most potent inhibition activity among the linear-chain fatty acids tested. Oleic acid was used as a representative of the *cis*-mono-unsaturated linear-chain fatty acids. From the Lineweaver–Burk plot in the absence of the compound, the K_m value of telomerase was 0.007 μ M with respect to the TS-A substrate (Figure 3A). By increasing the concentration of oleic acid from 0–40 μ M, the apparent K_m values were increased, whereas the apparent

Figure 1 Inhibitory effect of C_{10} to C_{24} linear-chain fatty acids ($100 \mu\text{M}$ each) on telomerase activity

The modified TRAP assay was employed to measure the telomerase activity after treatment with linear-chain fatty acids. **(A)** Saturated fatty acids. 10:0, capric acid; 12:0, lauric acid; 14:0, myristic acid; 16:0, palmitic acid; 18:0, stearic acid; 20:0, arachidic acid; 22:0, behenic acid; and 24:0, lignoceric acid. **(B)** *Cis*-unsaturated fatty acids. 12:1 Δ^9 cis, *cis*-9-dodecenoic acid; 14:1 Δ^9 cis, myristoleic acid; 16:1 Δ^9 cis, palmitoleic acid; 18:1 Δ^9 cis, oleic acid; 18:2 Δ^9 -12cis, linoleic acid; 18:3 Δ^9 -12-15cis, α -linolenic acid; 18:3 Δ^6 -9-12cis, γ -linolenic acid; 18:4 Δ^6 -9-12-15cis, stearidonic acid; 20:1 Δ^{11} cis, *cis*-11-icosenoic acid; 22:1 Δ^{13} cis, erucic acid; and 24:1 Δ^{15} cis, nervonic acid. **(C)** *Trans*-mono-unsaturated fatty acids. 14:1 Δ^9 trans, myristelaidic acid; 16:1 Δ^9 trans, palmitelaidic acid; 18:1 Δ^9 trans, elaidic acid; 18:2 Δ^9 -12trans, linoelaidic acid; 20:1 Δ^{11} trans, *trans*-11-icosenoic acid; and 22:1 Δ^{13} trans, brassidic acid. **(D)** C_{18} fatty acid derivatives. 18:1 Δ^9 cis-methyl, oleic acid methyl ester; 18:1 Δ^9 cis-ethyl, oleic acid ethyl ester; 18:1 Δ^9 cis-acetate, oleic acid acetate; 18:1 Δ^9 cis-alcohol, oleic acid alcohol.

$V_{\max}$ values were virtually constant, indicating that oleic acid competitively inhibited telomerase activity with respect to the TS-A substrate, with a K_i value of $3.06 \mu\text{M}$ as calculated from the replot (Figure 3B). In addition, using dNTPs as variable substrates, oleic acid showed non-competitive inhibition of telomerase (results not shown). Similar results were obtained with the other *cis*-unsaturated linear-chain fatty acids that inhibited telomerase activity (results not shown). Competitive inhibition kinetics of telomerase by fatty acids (i.e. competitive with the primer) appeared to be similar to that observed in DNA polymerase β [31,32], suggesting that oleic acid and the other fatty acids bind directly or interact with the oligonucleotide primer-binding groove on the telomerase, thereby decreasing its affinity for the primer, and the mechanism of binding of fatty acids to telomerase is similar to that on DNA polymerase.

When an excess of protein ($100 \mu\text{g/ml}$ BSA) or MS2 RNA was added to the reaction mixture, the telomerase inhibitory activity by oleic acid was not affected (results not shown). This suggested that, by competing with the primer without non-specific binding of the compounds with proteins or nucleotides, oleic acid could selectively bind to the oligonucleotide primer-binding site on telomerase. Therefore linear-chain fatty acids are not only selective inhibitors of human mammalian DNA polymerases and HIV-RT, but also of human telomerase via the same inhibitory mechanism.

3D structural modelling of linear-chain fatty acids

Oleic acid was the most potent telomerase inhibitor among the linear-chain fatty acids tested and competed with the substrate primer. Moreover, oleic acid could be considered to bind directly

Figure 2 Dose-response curves of linear-chain fatty acids on telomerase

(A) C_{14} to C_{24} mono-unsaturated fatty acids: myristoleic acid (14:1 $\Delta 9cis$, $\blacksquare$), palmitoleic acid (16:1 $\Delta 9cis$, $\bullet$), oleic acid (18:1 $\Delta 9cis$, $\circ$), *cis*-11-icosenoic acid (20:1 $\Delta 11cis$, $\blacktriangle$), erucic acid (22:1 $\Delta 13cis$, $\blacklozenge$) and nervonic acid (24:1 $\Delta 15cis$, $\blacktriangledown$). (B) C_{18} fatty acids: stearic acid (18:0, $\blacksquare$), oleic acid (18:1 $\Delta 9cis$, $\circ$), linoleic acid (18:2 $\Delta 9-12cis$, $\bullet$), α -linolenic acid (18:3 $\Delta 9-12-15cis$, $\blacktriangle$) and stearidonic acid (18:4 $\Delta 6-9-12-15cis$, $\blacklozenge$). The modified TRAP assay was employed to measure the telomerase activity.

to the primer-binding site on telomerase in a manner similar to the DNA polymerases, including HIV-RT, which is an RNA-dependent DNA polymerase. Telomerase is also an RNA-dependent DNA polymerase. Therefore inhibition of telomerase by oleic acid must occur in a similar 3D manner between the chemical structures of the enzyme protein and the fatty acid, as described previously for the DNA polymerases and fatty acids [31,32]. On the basis of previous computer modelling data [33], we have carried out a 3D-structural-modelling analysis of the linear-chain fatty acids and speculated on the mode of binding of telomerase with the fatty acid. The energy-minimized 3D computer modelling of the linear-chain fatty acids are shown in Figures 4(A) and 4(B). Oleic acid formed a U-shaped curve with a molecular width of 12.80 Å (where 1 Å = 0.1 nm) (Figure 4A). The hydrocarbon chain in the saturated and *trans*-unsaturated fatty acid molecules was linear and did not inhibit the telomerase activity (Figure 1A and 1C). Di- and more unsaturated fatty acids in the *cis* configuration had a decreased molecular width and did not form the U-shaped curve as seen in oleic acid, because the hydrocarbon chains in these fatty acids fold up at the position of the double bonds. This may provide an

Figure 3 Lineweaver-Burk plot of telomerase inhibition by oleic acid (18:1 $\Delta 9cis$)

(A) Telomerase reactions were performed by using the modified TRAP assay with K-562 lysate equivalent to 5000 cells and various concentrations of the TS-A substrate in the absence (●) or presence of 20 ($\square$) or 40 ($\blacktriangle$) μM oleic acid. (B) Replot of the slope versus the concentration of oleic acid. The inhibition constant (K_i) of oleic acid was 3.06 μM with respect to the TS-A primer, as determined from the x-axis intercept of the replots.

explanation as to why linoleic acid and polyunsaturated fatty acids showed weaker telomerase inhibitory activity compared with oleic acid (Figure 2B). Therefore the molecular width of the fatty acids appears to be important for telomerase inhibition. Furthermore, the molecular widths in the *cis*-mono-unsaturated linear-chain fatty acids are compared in Figure 4(C). The mono-unsaturated fatty acids, which consisted of hydrocarbon chain lengths from 16 to 20 carbons, inhibited telomerase activity (Figure 2A). The molecular widths in the C_{16} to C_{20} fatty acids were in the range of 11.53–14.26 Å. Oleic acid, the most potent telomerase inhibitor among the linear-chain fatty acids, was 8.50 Å long and 12.80 Å wide (Figure 4).

Information on the 3D structure of telomerase by X-ray crystallography and NMR analysis is unknown as yet. From the results above, however, the cubic structure of the linear-chain

Figure 4 Computer modelling of linear-chain fatty acids

(A) C_{18} fatty acids showing stearic acid (18:0, cyan), oleic acid (18:1 $\Delta 9cis$, orange), linoleic acid (18:2 $\Delta 9-12cis$, purple), α -linolenic acid (18:3 $\Delta 9-12-15cis$, yellow), γ -linolenic acid (18:3 $\Delta 6-9-12cis$, red) and stearidonic acid (18:4 $\Delta 6-9-12-15cis$, green). Both the carbon atom in the carboxy group and the hydrocarbon chain of their fatty acids were joined. (B) The view of the right-hand side of (A) is shown. (C) *Cis*-type mono-unsaturated fatty acids showing myristoleic acid (14:1 $\Delta 9cis$, cyan), palmitoleic acid (16:1 $\Delta 9cis$, orange), oleic acid (18:1 $\Delta 9cis$, purple), *cis*-11-icosenoic acid (20:1 $\Delta 11cis$, yellow), erucic acid (22:1 $\Delta 13cis$, blue) and nervonic acid (24:1 $\Delta 15cis$, red). Both the carbon atom in the carboxy group and the hydrocarbon chain of their fatty acids were joined. The distance between the carboxyl and hydrocarbon ends in the fatty acid molecular structures are 10.75, 11.53, 12.80, 14.26, 16.12 and 18.26 Å for myristoleic acid, palmitoleic acid, oleic acid, *cis*-11-icosenoic acid, erucic acid and nervonic acid respectively. The Figures were prepared using Insight II (Accelrys).

fatty acid-binding site on the telomerase protein could be assumed from the data reported previously [31,32] for those on the DNA polymerases, including HIV-RT. The inhibitory effects of the fatty acids on DNA polymerase activity occur by binding between the DNA polymerase protein and the fatty acid as a 1:1 complex [33]. The DNA template–primer-binding sites of the enzymes have a pocket which can bind to the fatty acid. If their fatty acid-binding site structures were three-dimensionally similar, the substrate primer-binding site of telomerase would also have a pocket which can bind to oleic acid, like the DNA polymerases, including HIV-RT [31,32]. The size of the pocket must have sufficient length and width to join to the oleic acid molecule. The pocket must consist of hydrophilic amino acids to bind to the substrate primer and a hydrophobic polypeptide sheet to bind to the hydrocarbon chain of the fatty acid. Moreover, the carboxy group of oleic acid must bind to the hydrophilic amino acid in the pocket in competition with the substrate primer, because the free carboxy group was important for telomerase inhibition

(Figures 1 and 3). These will be able to be predicted by X-ray crystallography and NMR analysis of the telomerase.

As described in the Introduction, telomerase is an attractive target for cancer chemotherapy [1,2]. Therefore clarification of the molecular mechanism of telomerase inhibition should provide the means to create an ideal artificial cancer chemotherapy agent. Our studies may provide insights into the mechanism of this effect, because *cis*-mono-unsaturated linear-chain (C_{16} – C_{20}) fatty acids selectively inhibited the activity of human telomerase and the mechanism of inhibition of telomerase by the fatty acids was almost the same as those of the DNA polymerases, including the RNA-dependent DNA polymerase HIV-RT, which have been extensively investigated by X-ray crystallography, NMR analysis and computer simulation modelling. Therefore the 3D structural information of the linear-chain fatty acids with regard to the inhibition described in the present study may facilitate computer design of new molecular probes capable of functioning as anti-neoplastic agents. For example, the sulphoquinovosylacetyl-

glycerols, fatty acid-containing sugars, which were initially found to be DNA polymerase inhibitors [35–40] and subsequently as an HIV-RT inhibitor by computer modelling (Y. Mizushima and K. Sakaguchi, unpublished work), could be potent telomerase inhibitors in fact, some of them were subsequently found to be so (Y. Mizushima and K. Sakaguchi, unpublished work).

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