Brief Articles

Molecular Design of Cholesterols as Inhibitors of DNA Polymerase α

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Received October 29, 2003

The triterpenoid structure is a promising motif for the molecular design of DNA polymerase inhibitors.[1] In this study, 2-(cholesteryloxy)acetic acid (**3**), 2-(cholestanyl)acetic acid (**7**), and 2-(stigmasteryl)acetic acid (**11**) were found to selectively affect only DNA polymerase α (pol.α). The presence of a carboxyl group at position 28 appears to be essential for the inhibition of the pol.α activity. With pol.α, these compounds acted by competing with the template-primer DNA and noncompetitively with the substrate.

Introduction

Some triterpenoids show potent inhibitory activities against mammalian DNA polymerases and DNA topoisomerases.[1] Some are effective against all those enzymes, while others are specific in their inhibition.[1] These results suggest that chemical modification of triterpenoids is a promising approach for the development of novel and specific inhibitors. In this study, to obtain useful information on molecular design, we carried out a systematic study of structure−activity relationships of chemically designed triterpenoids on DNA polymerases.

As described previously, one triterpenoid, the secondary bile acid lithocholic acid (LCA), selectively inhibits the activities of mammalian DNA polymerase α (pol.α) and β (pol.β), as well as DNA topoisomerases.[2,3] We found that pol.β and DNA topoisomerase II (topo II) had a similar three-dimensional (3D) structure, although their modes of action, amino acid sequences, and "a three-point interaction" between the small molecules and the pocket surface are markedly different from each other.[2] By means of 3D computer simulation, triangular pockets into which specific triterpenoids are to be incorporated were searched on pol.β and topo II.[2,3] The 3D structures of the deduced pockets were found to be very similar. Since LCA could also inhibit pol.α activity, we hypothesized the existence of a similar pocket somewhere on the pol.α surface. In addition, we already found that some particular triterpenoids specifically inhibited only pol.α activity (unpublished findings). These data suggest that there is some difference in the triangle structures among the pockets on the each enzyme so that the particular agents recognize them. Systematic studies using triterpenoid derivatives may facilitate the molecular design of inhibitors and the understanding of the mechanism on a molecular level, which eventually lead to the development of an anticancer agent.

Instead of LCA, we choose cholesterol as the triterpenoid skeleton because it is inexpensive and chemically more maneuverable.

Results and Discussion

Molecular Design of Cholesterol and Its Analogues as Inhibitors of DNA Polymerases and DNA Topoisomerases. As briefly described in Introduction, some triterpenoids selectively inhibit mammalian DNA polymerases and DNA topoisomerases.[1−4] One of them, fomitellic acid (FA),[4−6] was originally isolated as a nonprotein neurotrophic compound that induces neurite outgrowth in the human neuroblastoma cell line SH-SY5Y, which lacks signicant TRK family mRNA.[6] Another triterpenoid is the bile acid lithocholic acid (LCA) (Figure 1A). Both FA and LCA inhibit the activity of pol.α, pol.β, and topo II.[1−4] We proved that these triterpenoids bind to the DNA-binding domains of pol.β and topo II, competing with the template-primer DNA. On $^{1}H-^{15}N$ HMQC NMR analysis of pol.β with LCA, the 8 kDa domain of pol.β bound to LCA as a 1:1 complex with a dissociation constant (K_D) of 1.56 mM.[2] The binding region primarily comprised three amino acid residues (Lys60, Leu77, and Thr79) on the LCA interaction interface of pol.β.[1−3] Computational analyses using molecular simulation and surface analysis software indicated the 3D size of the LCA molecule to be 12.43 Å × 11.35 Å × 4.83 Å (Figure 1B). These dimensions are thought to be critical for pol.β inhibition.

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10.1021/jm030553v CCC: $27.50
Published on Web 08/11/2004

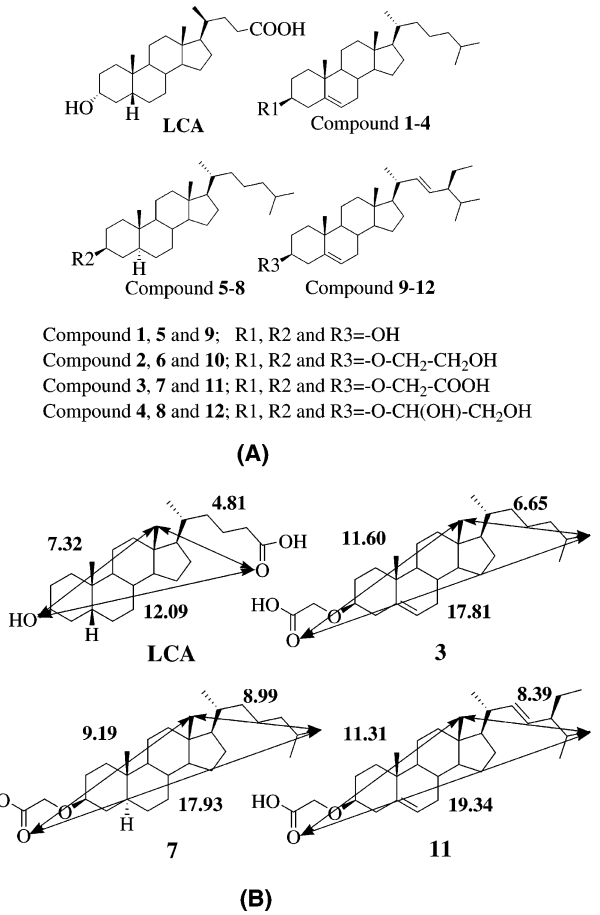

Figure 1. (A) Chemical structures of chemically modified cholesterols: LCA, lithocholic acid, **1**, cholesterol (CHE); **2**, ethylene glycol monocholesteryl ether (EG-CHE); **3**, 2-(cholesteryloxy)acetic acid; **4**, glycerol 1-monocholesteryl ether; **5**, cholestanol (CHA); **6**, ethylene glycol monocholestanyl ether (EG-CHA); **7**, 2-(cholestanyl)acetic acid; **8**, glycerol 1-monocholestanyl ether; **9**, stigmasterol (STE); **10**, ethylene glycol monostigmasteryl ether (EG-STE); **11**, 2-(stigmasteryl)acetic acid; **12**, glycerol 1-monstigmasteryl ether. (B) LCA, **3**, **7**, and **11**. Arrows indicate the lengths of each compound (Å).

In addition, a carboxyl group at position 29 is a key functional group for "a three-point interaction" between the small molecules and the pocket surface.[1−3] LCA must be incorporated into the pocket region comprising the three amino acid residues (Lys60, Leu77, and Thr79) of pol.β at the LCA interaction interface.[1−3] We could recognize "a three-point interaction" between the small molecules and the pocket surface (Lys720, Leu760, and Thr791) in the DNA-binding domain of topo II. Moreover, the interaction requires that the carboxyl group binds to Lys60 in pol.β or Lys720 in topo II, with precise 3D alignment. To clarify the situation with regard to pol.α, we evaluated the binding of various cholesterol derivatives and analyzed "a three-point interaction" between the small molecules and the pocket surface

Effects of Cholesterol and Its Analogues on the Activities of DNA Polymerases and DNA Topoisomerases. Table 1 shows the values of 50% inhibition (IC_{50}) of the cholesterol compounds **1**−**12** shown in Figure 1A against human pol.α, rat pol.β, and human topo II. As described previously,[3] LCA showed inhibition against pol.α, pol.β, and topo II with IC_{50} values of 45, 11, and 62 μM, respectively. IC_{50} values of **3**, **7**, and **11**

Table 1. IC_{50} Values (μM) of Modified Sterols on the Various DNA Polymerases[a]

	pol.α	pol.β	topo II
LCA	45	11	62
1	>500	>500	>500
2	>500	>500	>500
3	28	>500	>500
4	>500	>500	>500
5	>500	>500	>500
6	>500	>500	>500
7	20	>500	>500
8	>500	>500	>500
9	>500	>500	>500
10	>500	>500	>500
11	8	100	>500
12	>500	>500	>500

[a] Modified sterols were incubated with each enzyme (0.05 units). pol.α: human DNA polymerase α. pol.β: rat DNA polymerase β. topo II: human DNA topoisomerase II.

against pol.α were found to be 28, 20, and 8 μM, respectively (Table 1). The pol.α inhibitions by **3**, **7**, and **11** were much stronger than that by aphidicolin, which is often used as a specific inhibitor of the replicative DNA polymerases (pol.α, pol.δ, and pol.ϵ). Compounds **3**, **7**, and **11**, however, had no influence on the activities of pol.β and topo II (Table 1). These results demonstrate that compounds with a cholesterol skeleton could be designed as specific pol.α inhibitors.

Figure 1B shows 3D structures of the cholesterol compounds used and LCA. Although most of the cholesterol derivatives have almost the same triangle shape with a similar size (19 Å × 12 Å × 8 Å) (Figure 1B), **3**, **7**, and **11** with a carboxyl group at position 28 showed the inhibitory activity. Those derivatives lacking carboxylic group showed no inhibitory activity against pol.α. Although our previous results indicated that the 3D shape is important for the inhibition,[1−3] the present study revealed that the presence of the carboxyl group appears to be crucial in the cholesterol derivative. The shape (19 Å × 12 Å × 8 Å) greatly differed from the 3D size of LCA (12.43 Å × 11.35 Å × 4.83 Å) fitted in pol.β and topo II (Figure 1B), suggesting that the triangle pocket accepting triterpenoids in pol.β and topo II is too small to hold cholesterols. And this consideration leads to the assumption that pol.α has the alternative "a three-point interaction" that is capable of accepting the larger size of triterpenoids. LCA and FA inhibited the activities of pol.α, pol.β, and topo II with IC_{50} values of 27−58, 53−79, and 50 μM, respectively,[1−3] and they all have a carboxyl group at position 29, which corresponds to 28 in cholesterol. Triterpenoid derivatives in which the carboxylic acid moiety was removed completely lost the ability to inhibit pol.α.[1] Therefore, we speculated previously that the carboxylic acid moiety may play an important role in the inhibition of DNA polymerase and DNA topoisomerase and this also appears to be the case for cholesterol.

We tested the effects of reaction conditions on the pol.α inhibition to compare with those of FA and LCA described previously.[4,5] To determine the effects of a nonionic detergent on the binding of **3**, **7**, and **11** to pol.α, a neutral detergent, Nonidet P-40 (NP-40), was added to the reaction mixture at a concentration of 0.1%. In the absence of each of the compounds, the pol.α activity was taken as 100%. The pol.α inhibitory effects of **3**, **7**, and **11** at 100 μM were not affected by the

Table 2. Kinetic Analyses of DNA Polymerase α Inhibition by **3**, **7**, and **11**

	3	**7**	**11**
	DNA Template		
inhibition pattern	competitive	competitive	competitive
V_{max} (pmol/h)	2.97	2.63	3.63
K_i (μM)	18.5	13.8	27.9
	dNTP		
inhibition pattern	noncompetitive	noncompetitive	noncompetitive
K_m (μM)	4.21	2.63	1.43
K_i (μM)	3.75	7.84	11.2

addition of NP-40 to the reaction mixture (data not shown), suggesting that the binding interaction to the enzyme is hydrophilic. We also tested whether an excess amount of a substrate analogue, poly(rC) (100 μg/mL), or a protein, BSA (100 μg/mL), could prevent the inhibitory effects of **3**, **7**, and **11** to determine whether the effects are due to nonspecific adhesion to the enzyme or selective binding to a specific site. Poly(rC) and BSA showed little or no influence on the effects of **3**, **7**, and **11**, suggesting that the binding to pol.α occurs selectively.

None of the compounds **3**, **7**, and **11** showed any cytotoxic activity to DLD-1 cells (human colon cancer cell line) or NUGC-3 cells (human gastric cancer cell line) (data not shown). Their DNA polymerase inhibitory activity may thus be too weak to halt cell division.

Kinetic Mode of pol.α Inhibition by the Cholesterol Compounds 3, 7, and 11. Mode of the Binding among Them and pol.α. Next, to elucidate the mechanisms of inhibition, the extent of inhibition as a function of DNA template-primer or dNTP substrate concentrations was studied (Table 2). For kinetic analysis, poly(dA)/oligo(dT)[12−18] and dTTP were used as the template-primer DNA and dNTP substrate, respectively. Double reciprocal plots showed that **3**, **7**, and **11** inhibited pol.α activity competitively with the DNA template and noncompetitively with the dNTP substrate (Table 2). These results suggest that **3**, **7**, and **11** directly and competitively bind to the template-primer DNA-binding site of pol.α. These data differed from those for LCA, which inhibits pol.α activity noncompetitively with the DNA template and the dNTP substrate. This means that our novel compounds may provide direct clues to the 3D structure of the template-primer DNA-binding site of pol.α. As described above, the data indirectly support the assumption that the triangle pocket accepting LCA in pol.α may differ from that accepting the cholesterols.

We next investigated the interaction between pol.α and the active compounds **3**, **7**, and **11**. The template-primer DNA-binding protein activity of pol.α was analyzed by gel mobility shift assay. Figure 2 shows results with the M13 mp18 ssDNA−pol.α binding complex, separated into a major band and a faint band (Figure 2). The nature of the faint band is currently unclear. The bands may be self-primed linear DNA or, less likely, circular dimers. Pol.α bound to M13 mp18 ssDNA and was shifted in the gel (lanes 2−4, 8−10, and 14−16). In the binding assay, M13 mp18 ssDNA at 2.2 nmol of nucleotide was added with 25 pmol of the enzyme (lanes 2−6, 8−12, and 14−18). The molecular ratios of **3** (lanes

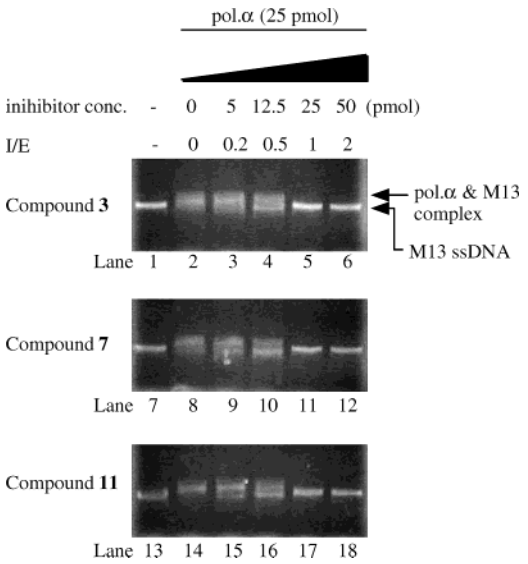

Figure 2. Gel mobility shift analysis. Gel shift analysis of binding between M13 single stranded plasmid DNA and DNA polymerase α (pol.α). M13 mp18 ssDNA (2.2 nmol, nucleotide) was mixed with purified proteins and the cholesterols. Lanes 1−6 contained pol.α at 0.2 nmol mixed with increasing concentrations of **3**, **7**, and **11**: 0.0, 0.2, 0.5, 1.0, 2.0 nmol. Samples were run on a 1.2% agarose gel in 0.1 M Tris-acetate (pH 8.3) containing 5 mM EDTA at 50 V for 2 h. Photographs of an ethidium bromide stained gel are shown.

1−6), **7** (lanes 7−12), and **11** (lanes 13−18) and the enzyme are shown as inhibitor-to-enzyme ratios (I/E) in Figure 2. When the I/E ratio was 1 or more, all compounds interfered with complex formation between M13 mp18 ssDNA and pol.α (lanes 5, 6, 11, 12, 17, and 18). At a ratio of 0.5, all effects disappeared, suggesting that one molecule of each compound competes with one molecule of M13 mp18 ssDNA and subsequently interferes with the binding of DNA to pol.α. The analysis indicated that all compounds acted by competing with the DNA template on pol.α; thus, all compounds directly bind to the DNA binding site of pol.α (Figure 2).

Relationship between the Size/Shape of Active Cholesterol Compounds and the Template-Primer DNA-Binding Site of pol.α. In pol.β, LCA appears to be inserted into the pocket region comprising three amino acid residues (Lys60, Leu77, and Thr79).[1−3] The size and the shape of LCA (12.43 Å × 11.35 Å × 4.83 Å), as shown in Figure 1B, exactly fit the three dimensions of the "a three-point interaction" between the small molecules and the pocket surface.[1,3] Although the 3D structure of pol.α is still unknown, it is thought to have such pocket for LCA interaction. Since the active compounds **3**, **7**, and **11** were almost same size (19 Å × 12 Å × 8 Å) (Figure 1B), the template-primer DNA-binding site of pol.α may also have "a three-point interaction" on the compound interactive surface. If so, the pocket in pol.α is larger to fit the size, 19 Å × 12 Å × 8 Å, than that in pol.β for LCA. The hydrophobic region (i.e., the backbone) and the hydrophilic region (i.e., carboxyl group or ketone group) in LCA are thought to bind to the hydrophobic sheet and the hydrophilic amino acids in the template-primer DNA-binding site of pol.β, respectively. The binding mode between the cholesterol and pol.α may be same as for the pol.β−LCA complex. The carboxyl group at position 28 appears to bind to the hydrophilic region in the pocket holding

cholesterols on pol.α. This might provide a clue or a key to elucidate the 3D microheterogeneity of the triterpenoid-accepting pockets on the template-primer binding sites among the DNA polymerases and the DNA topoisomerases, and consequently, the 3D structure of pol.α. Moreover, it may allow design of more potent DNA polymerase inhibitors using computer simulation.

Experimental Section

Chemical Synthesis of Cholesterol Analogues. Figure 1A shows chemical structures of lithocholic acid (LCA), cholesterol (**1**), cholestanol (**5**), stigmasterol (**9**), and other synthetic derivatives (**2**−**4**, **6**−**8**, and **10**−**12**) that were used in this study. Cholesterol derivatives **2**−**4** and cholestanol derivatives **8**−**10** were prepared according to the reported method.[1,8] Stigmasterol derivatives **10**−**12** were synthesized from stigmasteryl isomethyl ether. Treatment of stigmasteryl isomethyl ether with an excess amount of ethylene glycol in the presence of 10-camphorsulfonic acid (CSA) in CH_3CN afforded **10** in 85% yield. Oxidation of **10** by Jone's reagent gave the carboxylic acid **11** in 95% yield. Diol **12** was prepared by the reaction of stigmasteryl isomethyl ether with glycerol in the presence of CSA in CH_3CN in 83% yield.

Supporting Information Available: ^{1}H NMR, ^{13}C NMR, IR data, and biological evaluation of compounds listed in all figures and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

Note Added after ASAP Posting. This manuscript was released ASAP on 8/11/2004 with errors in the LCA structure in the table of contents graphic and in Figure 1. The correct version was posted on 8/24/2004.

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JM030553V