Inhibition Effect of New Farnesol Derivatives on All-*Trans*-Retinoic Acid Metabolism

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All-*trans*-retinoic acid (atRA) is a promising anticancer and antiwrinkle drug. However, its clinical application is limited because it is rapidly metabolized by the induced cytochrome P450 (P450). In this study, farnesol derivatives are proposed as new inhibitors to prevent P450-mediated metabolism. The farnesol derivatives were suc-farnesol and mal-farnesol, which were synthesized by the chemical conjugation of farnesol with succinic anhydride and maleic anhydride, respectively. The inhibition effects of farnesol, farnesoic acid, and farnesol derivatives on the atRA metabolism were evaluated in microsome and in AMC-HN-6 cells. In the microsome experiment, suc-farnesol and mal-farnesol strongly inhibited atRA metabolism at 10^{-4} mol/L concentration by as much as 61% and 77%, respectively. In the cell experiment, the inhibition effects of farnesol derivatives on the atRA metabolism showed similar tendency as the results in the microsome experiment, even if the effect was somewhat decreased. Effects of farnesoic acid and farnesol, however, were not significant. This research suggests that carboxylic end groups, such as atRA and hydrophobicity, might be important factors causing the higher inhibition effect, and that derivatization of farnesol can be 1 method to develop new inhibitors of atRA metabolism.

ALL-*TRANS*-RETINOIC acid (atRA) plays important roles in the regulation of proliferation and differentiation of epithelial tissues such as skin, bladder, lung, oral cavity, and mammary gland.[1-3] atRA is also effective in preventing second primary cancer for head and neck squamous cell carcinoma (HNSCC).[4] However, atRA is rapidly metabolized to inactive polar metabolites such as all-*trans*-4-hydroxy-RA and all-*trans*-4-oxo-RA.[5-7] This rapid metabolism of atRA is due to catabolism by a cytochrome P450 (P450), which is induced by atRA,[8-10] and this special P450 was recently cloned (CYP26).[11,12]. The evidence of P450 induction was shown in the acute resistance of atRA in a continuous oral administration.[13,14] That is, the half-life of atRA in the body is rapidly decreased with the repeated administration of atRA. Therefore, atRA application is quite limited, although atRA is a promising anticancer and an antiwrinkle drug.

To reduce the resistance of atRA, various imidazole derivatives, such as ketoconazole, liarozole, clotrimazole, miconazole, fluconazole, secnidazole, and metronidazole, have been studied since hydroxylation of atRA by P450 is inhibited by imidazole derivatives.[15-18] Among them, both ketoconazole and liarozole showed a potent inhibition effect on atRA metabolism. Ketoconazole reduced catabolism of atRA with several adverse reactions. Rigas et al[16] reported that common adverse effects of ketoconazole were headache, ear congestion, and hypertriglyceridemia. At higher doses, ketoconazole also showed serious mucocutaneous toxicity, nausea, and vomiting.[16] Liarozole has been reported to inhibit P450-mediated metabolism of atRA, thereby increasing the half-life of atRA. However, when atRA was administered repeatedly, liarozole could not completely inhibit atRA metabolism.[19]

In this study, the relationship of all-*trans* structure with carboxylic acid and the atRA metabolism was studied. Farnesol was used in this study because farnesol has all-*trans* structure and carboxylic acid at the end of its tail, like atRA. In this study, several farnesol derivatives were synthesized to examine the effects of modification of carboxylic end group and hydrophobicity of farnesol on atRA metabolism.

MATERIALS AND METHODS

Materials

atRA was obtained from Sigma Chemical Co (St Louis, MO). All-*trans*-[11, 12-^{3}H]-RA ([^{3}H]RA) (0.021 μmol/mL, 48.5 Ci/mmol) was obtained from Du Pont New England Nuclear (Boston, MA). Trans, trans-farnesol, chromium(VI) oxide, succinic anhydride, and maleic anhydride were purchased from Aldrich Co (Milwaukee, WI) and 4-dimethylaminopyridine (DMAP) was purchased from TCI (St Louis, MO). These materials were used without further purification. Sulfuric acid, ethyl ether, n-hexane, and isopropyl alcohol were used without further purification.

Synthesis of Farnesol Derivatives

Farnesoic acid was synthesized by the oxidation of primary alcohol of farnesol to carboxylic acid. Farnesol (10 g, 45 mmol) was dissolved in 200 mL of acetone and dropped into a sulfuric acid solution, which included an excess amount of chromium(VI) oxide (17 g, 180 mmol). After mixing for 24 hours, isopropyl alcohol was added to the reacting farnesol mixture to stop the reaction. The mixture was stirred for 30

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Submitted January 9, 2001; accepted April 19, 2001.

Supported by Grants No. HMP-96-G-2-32 and HMP-98-G-2-050-B of the Highly Advanced National Projects on the Development of Biomedical Engineering and Technology, Ministry of Health and Welfare, R.O.K. and partially supported by a Brain Korea 21 Project.

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0026-0495/01/5011-0001$35.00/0

doi:10.1053/meta.2001.27190

minutes to reduce the unreacted chromium(VI). The produced farnesoic acid was extracted from ethyl ether and purified by column chromatography.

Farnesol-succinic anhydride conjugate (suc-farnesol) was synthesized by reacting farnesol (2 g, 9 mmol) with succinic anhydride (1.08 g, 11 mmol) in acetone (30 mL). DMAP (1.31 g, 11 mmol) was used as a catalyst and the mixture was stirred for 24 hours at room temperature under nitrogen atmosphere. Hydrochloric acid (HCl) solution was added to the product to remove the remaining DMAP. The produced suc-farnesol was extracted from ethyl ether and purified by column chromatography. Farnesol-maleic anhydride conjugate (mal-farnesol) was synthesized by a similar method used in the synthesis of suc-farnesol. Farnesol (1.5 g, 6.7 mmol) was reacted with maleic anhydride (0.79 g, 8.04 mmol) in tetrahydrofuran (15 mL). Triethylamine (0.82 g, 8.04 mmol) was used as a catalyst, and the mixture was stirred for 24 hours at room temperature under nitrogen atmosphere. HCl solution was added to the product to remove the remaining triethylamine. The produced mal-farnesol was extracted from ethyl ether and purified by column chromatography. Molecular weights of farnesol derivatives were measured by gas chromatography–mass spectrometry (GC-MS). All of the farnesol derivatives were also analyzed by nuclear magnetic resonance (NMR) and infrared spectroscopy (FT-IR).

Cell Culture and Microsome Preparation

AMC-HN-6 cells, which could metabolize atRA rapidly by the induction of P450, were used in this study. The AMC-HN-6 cell line was established at Asan Medical Center (Seoul, Korea) from patients with HNSCC.[20] The cells were maintained in Eagle's minimum essential medium supplemented with 1% nonessential amino acid, 2 mmol/L L-glutamine, and 10% fetal bovine serum. At confluence, the cells were treated in the dark for 12 hours with 1 μmol/L atRA. All cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2. The cells were subcultured for a week at a split ratio of 1:10 using the trypsin/EDTA solution and checked regularly for mycoplasma contamination.

The confluent monolayer of cultured AMC-HN-6 cells was rinsed twice with ice-cold phosphate-buffered saline (PBS). The cells were treated with 0.25% trypsin for 10 minutes and then rinsed 4 times with ice-cold PBS by centrifugation for 5 minutes at 5,000$\times g$. The cells were resuspended in homogenization buffer (0.5 mol/L sucrose, 10 mmol/L Tris-HCl [pH 7.4], 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 0.1 μg/mL leupeptin, and 0.04 U/mL aprotinin) and homogenized in a Dounce glass tissue grinder (40 mL capacity, Kontes Glass Co, Vineland, NJ). The homogenate was diluted with an equal volume of 10 mmol/L Tris-HCl (pH 7.4) and 1 mmol/L EDTA, laid over the 0.5-vol homogenization buffer, and then centrifuged at 9,000 $\times$ *g* for 10 minutes at 4°C. The supernatant was centrifuged again at 100,000 $\times$ *g* for 45 minutes to remove cell debris, nuclei, and mitochondria. The pellet was resuspended in the storage buffer (0.25 mol/L sucrose, 10 mmol/L Tris-HCl, pH 7.4, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl floride, 0.1 μg/mL leupeptin, and 0.04 U/mL aprotinin) and stored at -20°C. The protein concentration was determined by the Coomassie Plus protein assay (Pierce Chemical, Rockford, IL).

Enzyme Assay

The enzyme assay was followed by the method described in our previous study.[20] Briefly, AMC-HN-6 cells (4×10^6) and microsomal proteins (0.5 mg) were assayed for enzymatic conversion of 33 nmol/L [^{3}H]-atRA (1.6 μCi/mL) to polar metabolites. The cells were incubated with [^{3}H]-atRA and various concentrations of a newly developed inhibitor in 600 μL PBS at 37°C for 60 minutes in the dark. On the other hand, microsomal proteins were incubated with [^{3}H]-atRA and various concentrations of the inhibitor in 600 μL buffer (1.5 mmol/L nicotinamide adenine dinucleotide phosphate [NADPH], 0.1 mol/L Tris-HCl [pH 7.4], 20 mmol/L sodium phosphate buffer [pH 7.0], 5 mmol/L $MgCl_2$, 0.15 mol/L KCl, and 10% glycerol) at 37°C for 60 minutes in the dark. The reaction was terminated by adding 600 μL chloroform and methanol (2:1), and this solution was centrifuged for 5 minutes at 5,000 $\times$ *g* to separate the phases. The lower organic phase containing retinoids was collected and dried with a stream of nitrogen in the dark. The dried extracts were resuspended in ethanol and analyzed by thin layer chromatography (TLC). The metabolic activity was expressed as a percentage of the total radioactivity of polar metabolites. In our previous report,[20] it was reported that the polar metabolites were all-*trans* 4-hydroxy retinoic acid, all-*trans* 18-hydroxy retinoic acid, and all-*trans* 4-oxo retinoic acid.

TLC Analysis

The dissolved sample (25 μL in ethanol) and standard retinoids were applied to TLC plates (LK6D silica gel) (Whatman, Hillsboro, OR) in the dark. TLC plates were developed for 90 minutes in a glass tank pre-equilibrated for 1 hour with 150 mL of developing solvent (hexane:ether:acetic acid = 90:60:1.5 vol:vol:vol). The glass tank also contained 1 sheet of solvent-saturated Whatman number 1 paper. TLC plates were air-dried for 5 minutes, and then retinoid standards were marked on the TLC plates. TLC plates were sprayed with [^{3}H] enhancer (DuPont-New England Nuclear) and then air-dried for 2 hours. The plates were exposed to X-OMAT film (Kodak, Rochester, NY) for 12 hours at -80°C to localize the retinoids. Using the developed film as a template, the radiolabeled atRA metabolites were scraped from the plate and mixed with 10 mL Bio Safe II (Research Products International, Mount Prospect, IL) and counted.

Cell Proliferation Assay

AMC-HN-6 cells (2×10^3) were plated onto 96-well dishes. Twenty-four hours later, various concentrations of farnesol derivatives were added to each well. The culture media were replaced after every 2 days with the similarly conditioned media and the total period of drug treatment of 9 days. Cell proliferation was measured using CellTiter 96 AQ_{ueous} Non-Reactive Cell Proliferation Assay (Promega, Madison, WI). In brief, 20 μL MTS/PMS solution (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium/phenazine methosulfate) was added per well and incubated at 37°C for 2 hours under the humidified atmosphere with 5% CO_2. The absorbance at 490 nm was recorded with an enzyme-linked immunosorbent assay (ELISA) plate reader.

RESULTS

Farnesoic acid was prepared by the oxidation of primary alcohol of farnesol to carboxylic acid, with 12% of the Jones Oxidative reaction yield. The prepared farnesoic acid was analyzed by ^{1}H NMR. The resonance peaks for C$\underline{H}_3$ and C$\underline{H}_2$-C$\underline{H}_2$ of farnesoic acid appeared in the range of 0.84 to 2.6 ppm. The ratio of peak areas of C=C$\underline{H}$ (t, 5.05 ppm) to C$\underline{H}$-COOH (s, 5.66 ppm) was the same with the ratio of their hydrogen numbers. The resonance peaks of C$\underline{H}_2$OH (d, 4.12 ppm) in farnesol disappeared in the spectrum of farnesoic acid. The triple peaks of C$\underline{H}$-CH_2OH at 5.38 ppm were shifted to the single peak at 5.66 ppm, which is identified as the peak of C$\underline{H}$-COOH. In the FT-IR spectrum of farnesoic acid, both stretching peaks of C=O and O-H were shown at 1685 cm^{-1} and 2500~3300 cm^{-1}, respectively. The molecular weight of prepared farnesoic acid was 236 d, which is the same value as the expected molecular weight.

Suc-farnesol was synthesized by the ring-opening reaction of succinic anhydride, which was initiated by the hydroxyl group

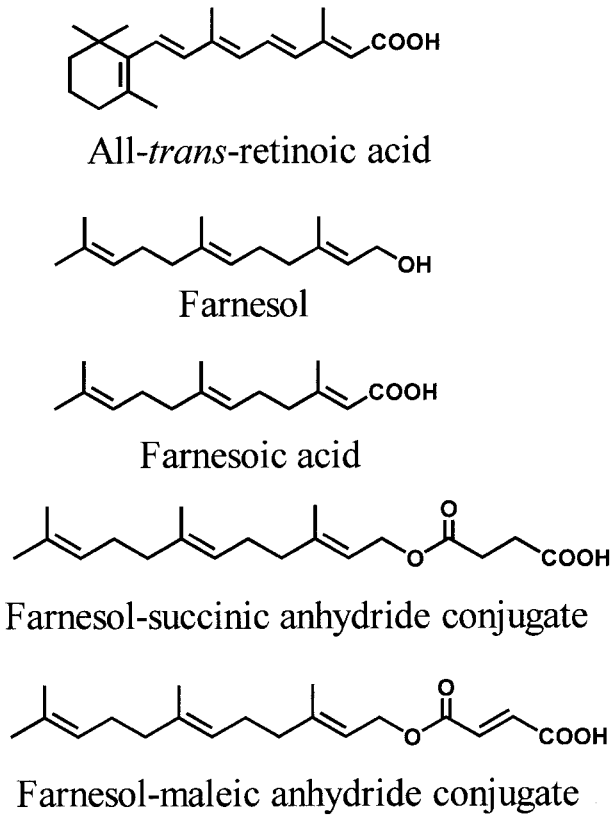

Fig 1. Chemical structures of retinoic acid and farnesol derivatives; (A) atRA, (B) farnesol, (C) farnesoic acid, (D) suc-farnesol, and (E) mal-farnesol.

of farnesol. The produced suc-farnesol has a carboxylic end group as shown in Fig 1. The yield of the reaction was 75%. In ^{1}H NMR spectrum, the resonance peaks for C$\underline{H}_3$ and C$\underline{H}_2$-C$\underline{H}_2$ were shown in the range of 1.54 to 1.97 ppm. The resonance peaks of C=C$\underline{H}$, C$\underline{H}$-CH$_2$COO, and C$\underline{H}_2$-COO were shown at 5.04, 5.28, and 4.55 ppm, respectively. These peaks are related to hydrogens of farnesol. On the other hand, the peak of C$\underline{H}_2$ of succinic anhydride (s, 2.9 ppm) was shifted to the peaks of C$\underline{H}_2$-C$\underline{H}_2$-COOH (t, 2.59 ppm), and the peaks of C$\underline{H}_2$OH (d, 4.12 ppm) of farnesol were shifted to the peaks of C$\underline{H}$-CH$_2$COO (d, 4.55 ppm). In the FT-IR spectrum, the stretching peak of C=O of ester group was shown at 1,747 cm^{-1}, and the stretching peak of carboxylic end group was shown at 2,500 to 3,300 cm^{-1}. The molecular weight of prepared farnesoic acid was 323 d, which was the same as the expected molecular weight.

Mal-farnesol was synthesized by the ring-opening reaction of maleic anhydride, which was initiated by the hydroxyl group of farnesol, thereby creating a carboxylic end group (Fig 1). In ^{1}H NMR spectrum, the resonance peaks for C$\underline{H}_3$ and C$\underline{H}_2$-C$\underline{H}_2$ of mal-farnesol were shown in the range of 1.54 to 1.97 ppm. The resonance peaks of C=C$\underline{H}$, C$\underline{H}$-CH$_2$COO, and C$\underline{H}_2$-COO were shown at 5.03, 5.33, and 4.71 ppm, respectively. These peaks were related to hydrogens of farnesol. The peak of C$\underline{H}$=C$\underline{H}$ of maleic anhydride was shown at 6.30 ppm as a double peak. In ^{13}C NMR spectrum, the carbon peaks of the

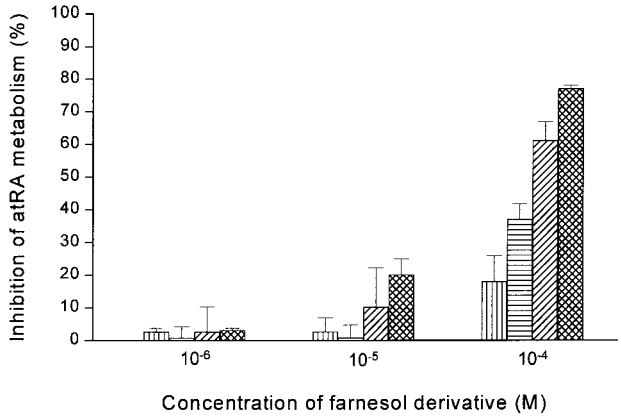

Fig 2. Effect of farnesol derivatives on the metabolism of atRA in microsome (n = 9, mean ± SD); ▤, farnesol; ▥, farnesoic acid; ▧, suc-farnesol; and ▨, mal-farnesol.

ester and carboxyl groups were shown at 166.7 and 169.9 ppm, respectively. In FT-IR spectrum, the stretching peak of C=O of the ester group was shown at 1,727 cm^{-1}, and the stretching peak of the O-H carboxylic end group was shown in the range of 2,500 to 3,300 cm^{-1}. The molecular weight of prepared mal-farnesol was 320 d, which was the same as the expected molecular weight.

To show the inhibition effect of farnesol derivatives on the atRA metabolism, the concentration of atRA was measured after microsomes were cultured for 1 hour in a buffer containing atRA and farnesol derivatives.[21] As the concentration of farnesol derivative was increased, the concentration of atRA increased; that is, the inhibition of atRA metabolism was increased as shown in Fig 2. Both suc-farnesol and mal-farnesol showed higher inhibition effects on the atRA metabolism than farnesol and farnesoic acid. The inhibition effects of suc-farnesol and mal-farnesol at 10^{-5} mol/L were as much as 10% and 20%, respectively, although they strongly inhibited atRA metabolism at 10^{-4} mol/L as much as 61% and 77%, respectively. On the other hand, farnesol and farnesoic acid did not

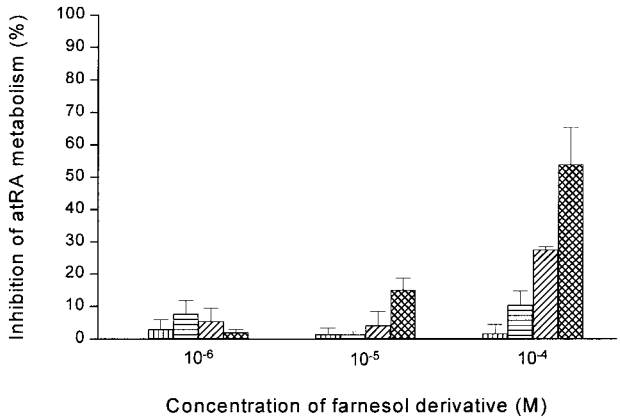

Fig 3. Effect of farnesol derivatives on the metabolism of atRA in AMC-HN-6 cells (n = 9, mean ± SD); ▤, farnesol; ▥, farnesoic acid; ▧, suc-farnesol; and ▨, mal-farnesol.

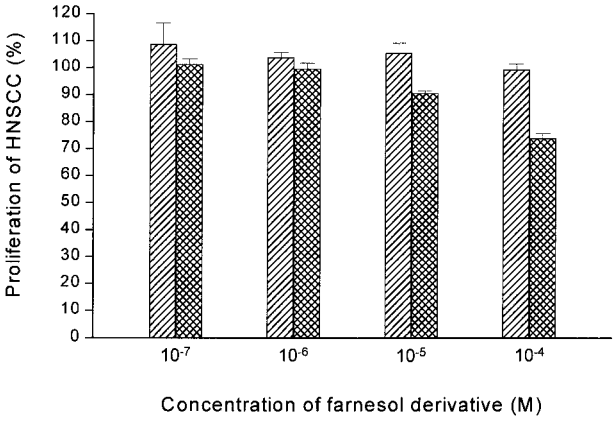

Fig 4. Effects of farnesol derivatives on proliferation of AMC-HN-6 cells (n = 9, mean ± SD); ▨, suc-farnesol and ▩, mal-farnesol.

show any inhibition effect on the atRA metabolism at 10^{-5} mol/L, but showed 37% and 18% inhibition at 10^{-4} mol/L, respectively.

In the cell experiment, the inhibition effects of farnesol derivatives on atRA metabolism showed a similar tendency as the results of the microsome experiment, even if the effect was somewhat decreased (Fig 3). The inhibition effects of suc-farnesol and mal-farnesol at 10^{-4} mol/L were significant as much as 28% and 54%, respectively. Farnesoic acid and farnesol did not show any significant effects on the inhibition of atRA metabolism.

The effects of farnesol derivatives on the proliferation of AMC-HN-6 cells, when AMC-HN-6 cells were incubated with farnesol derivatives continuously for 9 days, are shown in Fig 4. Suc-farnesol had no effect on cell proliferation when its concentration was in the range of 10^{-6} to 10^{-4} mol/L, and mal-farnesol showed 30% inhibition when its concentration was 10^{-4} mol/L. These results indicate that mal-farnesol itself might be weakly toxic to cells when the cell concentration is 10^{-4} mol/L.

DISCUSSION

Farnesoic acid, suc-farnesol, and mal-farnesol were all successfully synthesized with high product yields. Farnesoic acid was synthesized by oxidation of the hydroxy end group of farnesol to carboxylic end group. The molecular length of farnesoic acid is 16.2 Å, which is very similar to that of atRA (16.1 Å). The chain flexibility of farnesoic acid is better than that of atRA because the density of double bond in farnesoic acid is lower than that in atRA. Suc-farnesol has a relatively flexible carboxylic end group compared with farnesoic acid. Mal-farnesol has a double bond at the tail of the alkyl chain, and thus the structure of mal-farnesol is more similar to atRA than to suc-farnesol. However, mal-farnesol has a less flexible carboxylic end group compared with suc-farnesol. The lengths of suc-farnesol and mal-farnesol at the minimum energy state are 21.6 and 21.4 Å, respectively. These lengths are longer than that of atRA by as much as 135%.

In microsome experiments, both suc-farnesol and mal-farnesol significantly inhibited the metabolism of atRA above 10^{-5} mol/L. When farnesoic acid was compared with farnesol, the only difference in their structures was the presence of a carboxylic end group in farnesoic acid. Thus, this carboxylic end group might have affected the inhibition of metabolism because farnesoic acid, which has a carboxylic end group like atRA, showed higher activity than farnesol, which has a hydroxy end group. The carboxylic end group, however, was not the main factor in inhibiting metabolism. The difference between the inhibition in farnesoic acid and in farnesol was shown at such high concentrations as 10^{-4} mol/L, and both suc-farnesol and mal-farnesol showed higher activity in inhibiting the atRA metabolism than farnesoic acid.

The inhibition trend of farnesol derivatives in cells was similar to that in microsomes, even if the effect was somewhat decreased, because the cell membrane acts as a barrier against the permeation of farnesol derivatives into the cell. Both suc-farnesol and mal-farnesol permeate through the cell membrane faster than farnesol or farnesoic acid, because suc-farnesol and mal-farnesol are more hydrophobic owing to their higher number of carbon atoms. Thus, hydrophobicity may also be 1 of the factors causing the higher inhibition effect. Mal-farnesol has a stronger inhibitory effect than suc-farnesol, but suc-farnesol has no toxic effect below the concentration range of 10^{-4} mol/L. Therefore, suc-farnesol might be pharmacologically suitable. These results are preliminary data, and further studies will be performed to develop new compounds that are more suitable to the clinical applications.

Conclusively, we can draw upon the present results that carboxylic end groups like atRA and hydrophobicity might be important factors causing the higher inhibition effect, and that derivatization of farnesol may be 1 method to develop new inhibitors of atRA metabolism.

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