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RETINOID AGONIST ACTIVITIES OF SYNTHETIC GERANYL GERANOIC ACID DERIVATIVES

Hiroshi Araki, Yoshihiro Shidoji, Yasuhiro Yamada, Hisataka Moriwaki, and Yasutoshi Muto*

First Department of Internal Medicine, Gifu University School of Medicine, Tsukasa-machi 40, Gifu 500, JAPAN

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SUMMARY: Micromolar concentrations of 4,5- didehydro geranyl geranoic acid (GGA) were able to induce up-regulation of retinoic acid receptor-ß gene expression in human hepatoma-derived cell line, HuH-7, to the same extent as all-*trans* RA. In chloramphenicol acetyl transferase (CAT) assay with retinoic acid response element-ß, GGA and 4,5-didehydro GGA were both positive, but 2,3-dihydro GGA was negative, even though these GGA derivatives have been reported to be all potent ligands for cellular retinoic-acid-binding protein(CRABP). However, 10,11,14,15- tetrahydro- 4,5- didehydro GGA, a compound without any affinity for CRABP, transactivated CAT gene expression. On the other hand, only GGA and 4,5-didehydro GGA were active to induce CAT gene expression through retinoid X response element of cellular retinol binding protein, type II gene. We show for the first time that chemically synthesized acyclic organic acids are potential agonists of natural retinoids.

"Retinoid" is a general term that includes both the naturally occurring compounds with vitamin A activity and synthetic analogs, with or without biological activity, of retinol(1). IUPAC-IUB Joint Commission on Biochemical Nomenclature(1982) stated that, "Retinoids are a class of compounds consisting of four isoprenoid units joined in a head-to-tail manner. All retinoids may be formally derived from a monocyclic parent compound containing five carbon-carbon double bonds and a functional group at the terminus of the acyclic portion(2)." Thousands of retinoids have been so far developped, and they are all monocyclic or polycyclic compounds.

We have screened several tens of synthetic derivatives of geranyl geranoic acid consisting of four isoprenoid units joined in a head-to-tail manner and a carboxyl group at the tail terminus by using a binding assay for cellular retinoic acid-binding protein (CRABP)

*To whom correspondence should be addressed: FAX:+81-58-262-8484.

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(3). As a result of this screening, geranyl geranoic acid(GGA) and its 4,5-didehydro derivative (designated "acyclic retinoid"(4)) were found to be among the most potent ligands for CRABP, as strong as all-*trans* retinoic acid (RA). Acyclic retinoid has been found to reduce the incidence of skin tumors induced by 7,12-dimethylbenz[a] anthracene plus croton oil on mouse skin and block spontaneous hepatocarcinogenesis in C3H/HeNCrj male mice(5). Recently, we have also shown that acyclic retinoid induced up-regulation of albumin gene expression in human hepatoma-derived cell lines(6,7), and that the retinoid induced apoptosis in HuH-7 cells when fetal calf serum was removed from the culture medium(8). Although acyclic retinoid shows antitumor activities as strong as natural retinoids, its toxicity is much less than that of all-*trans* RA (LD_{50} in mouse; >6.25 g/kg *per os* for acyclic retinoid, 4.0 g/kg *per os* for all-*trans* RA(9), *in vitro* LD_{50} in PLC/PRF-5 cells; 86 μM for acyclic retinoid, 40 μM for all-*trans* RA(6)). The reason for this is probably by its metabolism in that a half of the radioactive acyclic retinoid injected was incorporated into the ß-position of triacylglycerol, similar to polyunsaturated fatty acids and the rest was rapidly degraded by ω- and ß-oxidations(Yoshimura T et al, unpublished results). Because of its less toxicity, acyclic retinoid is now being tested in phase II clinical trials with post-operative hepatoma patients.

After the discovery of nuclear retinoid receptors such as retinoic acid receptor (RAR) and retinoid X receptor (RXR)(reviewed in 10), most retinoids with biological activity, except for arotinoid, have been thought to be ligands for the receptors and to transactivate the putative target genes. However, to our knowledge, no acyclic compound have so far been reported as ligands for either RAR or RXR. In this paper, we addressed whether or not acyclic retinoid and other geranyl geranoic acid derivatives possess RA agonisit activity through retinoid receptors by induction of RAR-ß gene expression and chloramphenicol acetyl transferase (CAT) assay.

Materials and Methods

Chemicals and DNAs: Several synthetic polyisoprenoic acids shown in Fig. 2 were supplied from Eisai Co. Tokyo. All-*trans* RA was purchased from Sigma Co. and 9-*cis* RA was a generous gift from Kuraray Co. Okayama. pRARßCAT conveying CAT gene recombinated with retinoic acid response element (RARE-ß) of RAR-ß gene, pCRBPIICAT consisting of CAT gene and retinoid X response element (RXRE) of human cellular retinol-binding protein, type II (CRBP II) gene and pRShRARß containing human RAR-ß cDNA (Eco RI fragment) were from Dr. R.M. Evans, The Salk Institute, La Jolla, California.

Northern Blot Hybridization: After 24-hr treatment of human hepatoma-derived cell line, HuH-7 cells, cultured with alpha-MEM containing 10 % fetal calf serum, with all-*trans* RA or 4,5-didehydro GGA(0.1~10 μM), total RNA was extracted by acid-guanidium-phenol-chloroform method(11). Twenty μg of total RNA were applied onto 1.2 % agarose gel in 1 % formaldehyde. The separated RNAs blotted on a nylon membrane (Hybond N, Amersham, UK) were hybridized with a probe of RAR-ß cDNA labelled with digoxigenin-UTP. Chemiluminescent detection was performed according to Blum et al (12).

CAT Assay: CAT gene plasmid, either pRARßCAT or pCRBPIICAT, was transfected to 70% confluent HuH-7 cells (5 x 10^6 cells/well) by electroporation with Bio-Rad gene pulser. Conditions of electroporation were volume of cell suspension: 0.5 ml, electrode distance: 0.4 cm, voltage: 0.22 kV, capacitor: 960 µF, and time constant: 23 ~ 25 msec. The pulsed cells outgrew in alpha-MEM containing 10 % fetal calf serum overnight. Then, the attached cells were treated for 16 hours with 1 µM all-*trans* RA, 9-*cis* RA, or synthetic geranyl geranoic acid derivatives. Total cell extracts prepared from the treated cells were analyzed by CAT assay with [dichloroacetyl-1,2-^{14}C]-chloramphenicol (1.850 MBq/mmol, CAT assay grade, New England Nuclear,USA).

Results

All-*trans* RA induced up-regulation of RAR-ß expression in human hepatoma-derived cell line, HuH-7 cells (Fig. 1, *lanes* 5-7). Synthetic 4,5-didehydro GGA was also active to induce up-regulation of RAR-ß expression to the same extent as all-*trans* RA (Fig. 1, *lanes* 2-4).

To determine whether or not the GGA derivatives act through retinoic acid response element-ß (RARE-ß) residing at the 5'-flanking region of RAR-ß gene, sub-confluent HuH-7 cells were transfected with pRARßCAT, which contains the RARE-ß sequence fused to the CAT reporter gene. After transfection, the cells were treated for 16 hr with 1 µM RA or GGA derivatives. All-*trans* RA treatment produced a 6-fold increase in CAT activity. Both GGA and 4,5-didehydro GGA induced CAT gene expression to the same extent as all-*trans* RA as shown in Fig. 2. Two other GGA derivatives of 4,5-didehydro-10,11-dihydro GGA and 10,11,14,15-tetradehydro-4,5-didehydro GGA were positive to induce CAT enzyme activity, but 2,3-dihydro, 4,5,8,9-tetrahydro, and 14,15-dihydro derivatives of GGA were found negative in this CAT assay (Fig. 2).

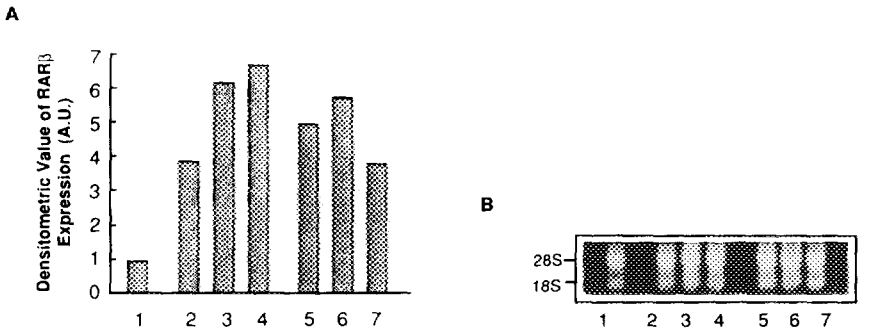

Fig. 1. Induction of RARß expression by all-*trans* RA or 4,5-didehydro GGA in HuH-7 cells.

RARß expression was analysed by Northern blot hybridization 24 hrs after the retinoid treatment . Lanes 1; control, 2~4; 4,5-didehydro GGA(2; 0.1 µM, 3;1.0 µM, 4; 10.0 µM) and 5~7; all-*trans* RA(5; 0.1 µM, 6;1.0 µM, 7; 10.0 µM). A: Densitometric analyses were done for Northern blots of RARß. B: Ethydium bromide-staining of ribosomal RNA blotted to the nylon membrane.

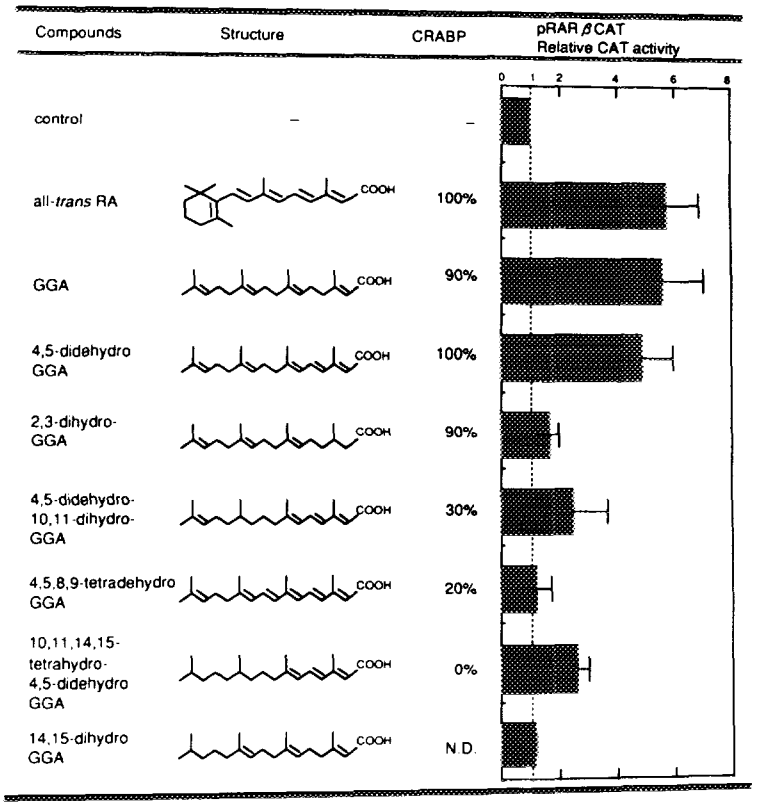

Fig. 2. CAT assay for synthetic GGA derivatives with RARE/CAT plasmid.
CAT assay was performed as described in *Materials & Methods*. The average number of relative intensity in triplicate to control in densitometric values were plotted with standard error. The relative intensity of binding activity of synthetic GGAs to all-*trans* RA for CRABP was referred from Muto, Y. et al.(3). N.D.; not determined.

In CAT assay with pCRBPIICAT plasmid, consisting of RXRE residing on 5'-flanking region of CRBP II gene, only two derivatives, GGA itself and 4,5-didehydro GGA were able to give positive spots on TLC plates to the same degree as 9-*cis* RA. Other GGA derivatives were negative as shown in Fig. 3.

Discussion

In the present report, we showed for the first time that chemically synthesized acyclic geranyl geranoic acid derivatives were potent agonists of all-*trans* or 9-*cis* RA by CAT assay.

In the first place, we observed the induction of RAR-ß gene expression by 4,5-didehydro GGA derivative in human hepatoma-derived cell line, HuH-7 cells. Human RAR-ß gene possesses its own retinoic acid response element (RARE-ß) so that the up-regulation of

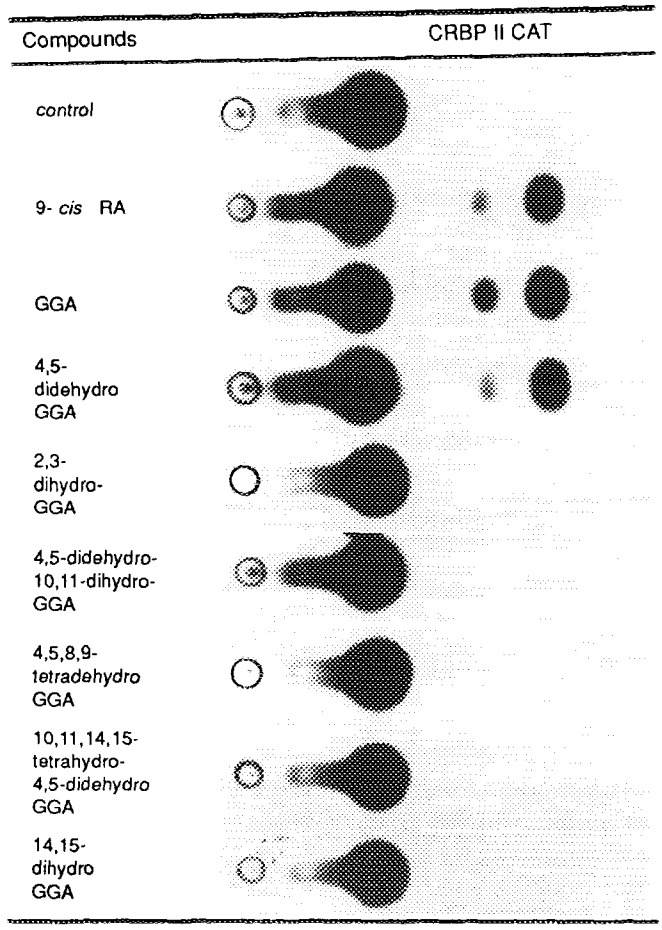

Fig. 3. CAT assay for synthetic GGA derivatives with RXRE/CAT plasmid. CAT assay was performed as described in *Materials & Methods.*

the RAR-ß gene expression is a sort of indicator for "retinoid function"(10). In this context, we could clearly show apparent "retinoid function" of the GGA derivative. Furthermore, as reported previously in the same cell system, this GGA derivative also showed retinoid-like functions such as down-regulations of c-*myc* and α-fetoprotein gene expression(7). These genomic effects of the GGA derivative were considered to occur through nuclear retinoid receptors.

Therefore, we tested several GGA derivatives, listed in Fig. 2, by CAT assays with retinoic acid response element (RARE) or retinoid X response element (RXRE). Among GGA derivatives, we would like to emphasize that 4,5,8,9-tetradehydro GGA was defective to induce CAT gene through RARE, suggesting that a length of all-*trans* conjugation system may be critical in RAR-mediated gene activation. Interestingly, 2,3-dihydro GGA has been shown a potent ligand for CRABP, which means that the allylic structure is not

required for binding to CRABP, however, this structure is essential for ligand activity to RAR.

In sharp contrast, RXRE/CAT assay required a relatively strict specificity for GGA derivatives. Only two synthetic compounds of GGA and 4,5-didehydro GGA were positive in the CAT assay. In terms of dual action with either RARE or RXRE, these two GGA derivatives resemble 9-*cis* RA. However, unlike 9-*cis* RA, these two GGA derivatives have been shown to bind to CRABP (3). Therefore, these GGA derivatives are recognized as unique retinoids both functionally as well as structurally.

GGA derivatives may occur in animal cell system. Geranyl geranoic acid may be obtained by dephosphorylation and oxidation of geranyl geranyl pyrophosphate, a common intermediate of polyprenol, dolichol and ubiquinone biosynthesis pathways(13). On the other hand, GGA and/or 4,5-didehydro GGA can be produced by a central cleavage and consecutive oxidation of naturally occurring acyclic carotenoids such as phytoene, phytofluene, zeta-carotene, neurosporene and zeacarotene.

Finally, we would like to mention that 10,11,14,15- tetrahydro-4,5-didehydro GGA is one of the candidates for differentiation therapy of acute promyelocytic leukemia (APL). During all-*trans* RA therapy for APL, RA-resistant patients show elevation of cellular levels of CRABP which may sequester functional all-*trans* RA(14). However, this GGA derivative showed all-*trans* RA agonist activity and is not a ligand for CRABP. Furthermore, it is the most important for clinical application that GGA derivatives are extremely less toxic than both natural retinoids and synthetic retinoids so far developped.

In any event, we have shown that some GGA derivatives are potent agonist of natural retinoids, although we can not still exclude the possibility that these two GGA derivatives may influence intracellular metabolism of endogenous retinoids and consequently show apparent "retinoid functions".

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