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A NEW SYNTHETIC ETHER AMINOPHOSPHOGLYCERIDE EXHIBITS PARTIAL MODULATOR ACTIVITY TOWARDS THE GLUCOCORTICOID RECEPTOR

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Summary: Modulator is an endogenous low-molecular weight regulator of both glucocorticoid and mineralocorticoid receptors, as well as protein kinase C. Analogs of the putative modulator structure have been synthesized. These compounds include 1-O-(3'-carboxypropyl) or (5'-carboxypentyl)-L-glycero-3-phospho-L-serine or L-threonine, and the D-glycerol stereoisomers. These compounds were tested for *in vitro* modulator activity using the glucocorticoid-receptor complex activation inhibition and steroid-binding stabilization assays. One of the ether phosphoglycerides, 1-O-(5'-carboxypentyl)-L-glycero-3-phospho-L-threonine (H-GPT-1), partially inhibited steroid-receptor complex activation in a dose-dependent manner. However, none of the other compounds exhibited any modulator activity towards the glucocorticoid-receptor complex. Like modulator, H-GPT-1 did not inhibit activated glucocorticoid-receptor complex binding to DNA-cellulose. Surprisingly, in contrast to modulator, H-GPT-1 partially inhibited unoccupied receptor steroid-binding in a dose-dependent manner. These results suggest that although modulator is not exactly mimicked by this compound, H-GPT-1 is the first synthetic organic molecule to exhibit some modulator activity towards the glucocorticoid receptor.

The formation of the cytoplasmic glucocorticoid-receptor complex initiates a series of events which ultimately leads to the regulation of specific gene transcription (1, 2). The first of these events after steroid-binding is known as activation (or transformation) (2, 3). Activation involves a topological change in the unactivated receptor structure, which results in a decrease in the size and acidity of the receptor, and an increase in receptor DNA-binding ability. At least some of these changes in receptor structure are due to the dissociation of the 90,000 dalton heat-shock protein from the unactivated receptor complex (4, 5). Glucocorticoid-receptor complex activation has been shown

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to occur *in vivo* (6, 7), and it is thought to be the rate-limiting step in the formation of the receptor-nucleus complex (8). Subsequent events after activation include the translocation of the activated hormone-receptor complex to the nucleus, the binding of the activated receptor to discrete sites on the chromatin, and the eventual enhancement or repression of distinct mRNA synthesis (1, 2).

Modulator is an endogenous low-molecular weight inhibitor of glucocorticoid-receptor complex activation that was originally identified in rat liver cytosol (9-11). This receptor activation inhibitor also stabilizes the steroid-binding ability of the unoccupied (or hormone-free) form of the glucocorticoid receptor (2, 4). Exogenous sodium molybdate (Na_2MoO_4) has similar biological activities towards the steroid hormone receptors as modulator (2, 4). In 1988, the apparent purification and initial characterization of modulator from rat liver cytosol was described. (12, 13). Structural analysis of apparently purified modulator by chemical assays, ultra-violet spectroscopy, infra-red spectroscopy, nuclear magnetic resonance spectroscopy, and mass spectrometry suggested that modulator was a novel ether aminophosphoglyceride (Table 1).

Recently, the isolation of two new modulator isoforms from rat liver cytosol was reported (14). These isoforms were called "modulator-1" and "modulator-2" based on the elution of the molecules from a giant Sephadex G-15 gel-filtration column. The two modulators act synergistically to inhibit steroid-receptor complex activation. More recent work demonstrates that the modulators also regulate mineralocorticoid receptor function (15) and stimulate protein kinase C activity (16).

Since the modulators appear to be important and novel biomolecules, the unambiguous structural determination and eventual chemical synthesis of these endogenous regulators are pre-requisites for a compendium of future studies. Steps have recently been taken towards the development of a synthetic modulator-like compound. The first series of novel synthetic ether phosphoglycerides included 1-O-(6'-carboxyhexyl)-rac-glycero-3-phospho-L-serine and related analogs; unfortunately, none of those compounds exhibited modulator activity (17).

This report describes the biological characterization of a second series of synthetic compounds including 1-O-(3'-carboxypropyl) or (5'-carboxypentyl)-L-glycero-3-phospho-L-serine or L-threonine, and the D-glycerol stereoisomers (Table 1). In contrast to the first group of compounds, the present series of ether phosphoglycerides were synthesized in a stereospecific manner (18, 19). These phospholipid analogues were designed to asses if variations of (i) the carboxyalkyl chain-length, (ii) the glycero stereocenter, and (iii) the nature of the amino acid substituent would result in a biologically active modulator derivative(s). One of these compounds, 1-O-(5'-carboxypentyl)-L-glycero-3-phospho-L-threonine (H-GPT-H1), partially inhibited glucocorticoid-receptor complex activation in a dose-dependent manner. However, none of the other compounds, including the 1-O-(5'-carboxypentyl)-D-glycero-3-phospho-L-threonine stereoisomer (H-GPT-H2), exhibited any modulator activity towards the steroid-receptor complex. These results suggest that although modulator

is not exactly mimicked by any one of these synthetic ether phosphoglycerides, H-GPT-H1 is the first synthetic organic molecule to exhibit some modulator activity towards the glucocorticoid receptor.

EXPERIMENTAL PROCEDURES

Preparation of Stock Solutions of Synthetic Modulator Analogs: The novel ether aminophosphoglycerides were synthesized and purified to apparent homogeneity as previously described (18, 19). The dried compounds were resuspended in 50 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 100 mM KCl, pH 7.4 buffer at a concentration of ~7.5 mg/ml (15-20 mM); these stock solutions were stored frozen at -20°C. All aqueous buffers were made with high-performance liquid chromatography grade/0.2 μm filtered water, which was produced by a NANOpure Water System (Barnstead/Thermolyne). All of the compounds were readily soluble at this concentration in the aqueous solution. The approximate pH for this stock solution was pH 6-7 as determined by indicator paper. The final pH for the tested concentrations of the compounds in the glucocorticoid receptor assays was pH 7-8.

Cytosol and Steroid-Binding: Liver cytosol was prepared from adrenalectomized male Sprague-Dawley rats (Buckshire Farms) as previously described (20), except that 50 mM KH_2PO_4, 5 mM dithiothreitol, 10 mM monothioglycerol, 20 mM Na_2MoO_4, 1 mM phenylmethylsulfonyl fluoride, pH 7.0 buffer was used. Steroid-binding was performed by incubating aliquots of rat liver cytosol with 100 nM [^{3}H]triamcinolone acetonide (9α-fluoro-11β,21-dihydroxy-16α,17-[1-methylethylidene-bis(oxy)]pregna-1,4-diene-3,20 dione; 49.4 Ci/mMol, New England Nuclear) for 2-3 hr on ice in the presence and absence of 500-fold molar excess radioinert triamcinolone acetonide in order to determine the non-specific steroid-binding, which was ≤ 5% of the total binding. The total steroid-binding was typically 60,000-80,000 disintegrations per min per 50 μl.

Hydroxylapatite and DNA-Cellulose Binding: Total [^{3}H]triamcinolone acetonide-receptor complexes were determined by hydroxylapatite binding, and activated [^{3}H]triamcinolone acetonide-receptor complexes were measured by DNA-cellulose binding as previously described (14, 21). The percent binding to DNA-cellulose was calculated by dividing the DNA-cellulose disintegrations per min by the hydroxylapatite disintegrations per min and multiplying by 100.

Activation Inhibition Assay: The *in vitro* glucocorticoid-receptor complex activation inhibition and steroid-binding stabilization assay was performed essentially as described previously (12-14). The percent binding to DNA-cellulose relative to control was calculated as previously described (13). The background binding of unactivated receptors to DNA-cellulose was ≤ 2%, while the binding of activated receptors to DNA-cellulose was 60-70%.

Activated Receptor DNA-Cellulose Binding Inhibition Assay: The *in vitro* activated glucocorticoid-receptor complex DNA-cellulose binding inhibition assay was performed essentially as described previously (17).

Unoccupied Receptor Steroid-Binding Stabilization Assay: The *in vitro* unoccupied glucocorticoid receptor steroid-binding stabilization assay was performed essentially as described previously (12-14). The percent specific [^{3}H]triamcinolone acetonide-binding to hydroxylapatite relative to control was calculated as previously described (13).

RESULTS: Table 1 summarizes the chemical structures and the biological effects of the six stereospecific synthetic putative modulator phosphoglycerides. These compounds include 1-O-(3'-carboxypropyl) or (5'-carboxypentyl)-L-glycero-3-phospho-L-serine or L-threonine, and the D-glycerol stereoisomers (18, 19). Four of the ether phosphoglycerides were isolated as disodium salts (B-GPS-A1, B-GPS-A2, and H-GPS-A1), while two were isolated as free-acids (H-GPT-H1 and H-GPT-H2). Like modulator, all of these compounds were readily soluble in aqueous solutions at a concentration of 15-20 mM.

The synthetic compounds were tested for the ability to inhibit *in vitro* glucocorticoid-receptor complex activation (Figure 1). Neither H-GPS-A1, nor the other L-serine containing phosphoglycerides (B-GPS-A1 and B-GPS-A2; Table 1), inhibited steroid-receptor complex activation. However, like modulator, the L-threonine containing compound H-GPT-H1 did appear to inhibit glucocorticoid-receptor complex activation in a dose-dependent manner. At a concentration of 3-4 mM, H-GPT-H1 inhibited receptor activation by 35-40%; higher concentrations of this compound (up to ~8 mM) did not have a further effect on receptor activation (data not shown). However, H-GPT-H1 was not as potent as sodium molybdate, which inhibited glucocorticoid-receptor complex activation by 50% at a concentration of ~1 mM. When the D-glycerol stereoisomer of H-GPT-H1 was tested (H-GPT-H2), it did not inhibit steroid-receptor complex activation. Thus, H-GPT-H1 seemed to significantly inhibit

Table 1

Effects of Synthetic Putative Modulator Phosphoglycerides on Glucocorticoid Receptor Activity

Chemical Structure	Compound	Modulator Activity
Modulator	1-O-(3'-Carboxypropyl to 7'-Carboxyheptyl)-L-Glycero-3-Phospho-L-(γ-aminothreonine)	+
B-GPS-A1	1-O-(3'-Carboxypropyl)-L-Glycero-3-Phospho-L-Serine, 2 Na	-
B-GPS-A2	1-O-(3'-Carboxypropyl)-D-Glycero-3-Phospho-L-Serine, 2 Na	-
H-GPS-A1	1-O-(5'-Carboxypentyl)-L-Glycero-3-Phospho-L-Serine, 2 Na	-
H-GPT-H1	1-O-(5'-Carboxypentyl)-L-Glycero-3-Phospho-L-Threonine, FA	+/-
H-GPT-H2	1-O-(5'-Carboxypentyl)-D-Glycero-3-Phospho-L-Threonine, FA	-

Refer to Bodine and Litwack (12, 13) for a description of the original purification and characterization of modulator from rat liver cytosol. The stereochemistry for modulator was assumed to be of the L-stereoisomer. The putative modulator compounds were stereospecifically synthesized and purified as previously described (18, 19). Nomenclature: B, butanoic acid; H, hexanoic acid; GPS, glycerophosphoserine; GPT, glycerophosphothreonine; A, anion; H, acid; 1, first stereoisomer; 2, second stereoisomer. Key: 2 Na, disodium salt; FA, free-acid; +, complete modulator activity; -, no modulator activity; +/-, partial modulator activity.

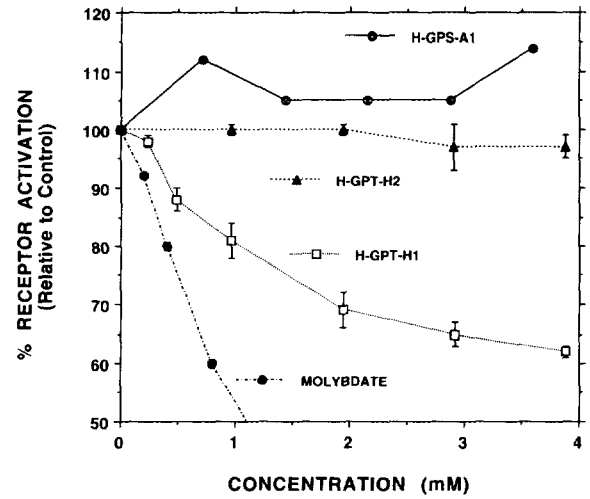

Figure 1. H-GPT-H1 inhibits glucocorticoid-receptor complex activation in a stereospecific manner. The synthetic novel phosphoglycerides and sodium molybdate were tested for the ability to inhibit *in vitro* glucocorticoid-receptor complex activation as described in "Experimental Procedures". Refer to Table 1 for the structures of the synthetic compounds. The data for H-GPS-A1 are presented as means of duplicates, while the rest of the data are presented as the mean ± SE, n=3-7. The Student's t-test p value for the difference between H-GPT-H2 and H-GPT-H1 at 3.9 mM was $p<0.005$.

glucocorticoid-receptor complex activation in a stereospecific manner ($p < 0.005$ when compared to H-GPT-H2 at 3.9 mM).

In order to confirm that H-GPT-H1 was actually inhibiting glucocorticoid-receptor complex activation, and not simply blocking activated steroid-receptor complex binding to DNA-cellulose, the experiment depicted in Figure 2 was performed. The

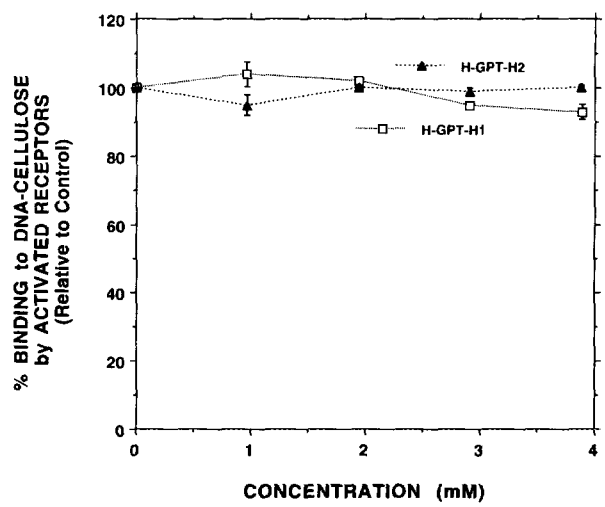

Figure 2. H-GPT-H1 does not inhibit activated glucocorticoid-receptor complex DNA-cellulose binding. The synthetic novel phosphoglycerides were tested for the ability to inhibit *in vitro* activated glucocorticoid-receptor complex DNA-cellulose binding as described in "Experimental Procedures". The data are presented as the mean ± SE, n=3-4.

results of this experiment show that neither H-GPT-H1 nor H-GPT-H2 inhibited *in vitro* activated receptor DNA-cellulose binding. This observation is important, because several naturally occurring phosphoglycerides have been previously shown to inhibit activated glucocorticoid-receptor complex DNA-cellulose binding (13, 17). Therefore, H-GPT-H1 is a stereospecific inhibitor of glucocorticoid-receptor complex activation. Modulator and molybdate do not inhibit activated steroid-receptor complex DNA-cellulose binding either (13).

Finally, the synthetic novel phosphoglycerides were tested for the ability to stabilize *in vitro* unoccupied glucocorticoid receptor steroid-binding (Figure 3). Neither H-GPS-A1, nor the other L-serine containing compounds (B-GPS-A1 and B-GPS-A2; Table 1), stabilized unoccupied receptor hormone-binding. Surprisingly, and in contrast with modulator and molybdate, H-GPT-H1 did not stabilize unoccupied glucocorticoid receptor steroid-binding, but instead it inhibited hormone-binding in a dose-dependent manner. At a concentration of 3-4 mM, H-GPT-H1 inhibited unoccupied receptor steroid-binding by 30-40%. When H-GPT-H2 was tested, it did not inhibit glucocorticoid-binding. Thus, H-GPT-H1 seemed to significantly inhibit unoccupied glucocorticoid receptor hormone-binding in a stereospecific manner ($p < 0.02$ when compared to H-GPT-H2 at 3.9 mM). Once again, H-GPT-A1 had no effect

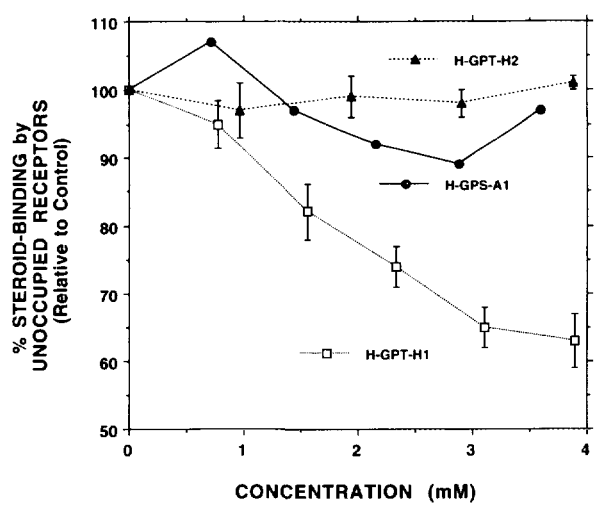

Figure 3. H-GPT-H1 inhibits unoccupied glucocorticoid receptor steroid-binding in a stereospecific manner. The synthetic novel phosphoglycerides were tested for the ability to stabilize *in vitro* unoccupied glucocorticoid receptor steroid-binding as described in "Experimental Procedures". The data for H-GPS-A1 are presented as means of duplicates, while the rest of the data are presented as the mean ± SE, n=3. The Student's t-test p value for the difference between H-GPT-H2 and H-GPT-H1 at 3.9 mM was $p<0.02$. Twenty-five mM sodium molybdate (not shown) stabilized unoccupied glucocorticoid receptor steroid-binding 187 ± 10% (mean ± SE, n=4). The percent specific [^{3}H]triamcinolone acetonide-binding to hydroxylapatite relative to control was calculated as previously described (13), except that the heat-treated buffer control was set equal to 100%.

on unoccupied receptor steroid-binding (Table 1). Modulator and molybdate stabilize unoccupied receptor steroid-binding (13).

DISCUSSION: The results described in this report present evidence that 1-O-(5'-carboxypentyl)-L-glycero-3-phospho-L-threonine (H-GPT-H1) exhibits some modulator-like effects towards the glucocorticoid receptor (Table 1). This new synthetic ether aminophosphoglyceride partially inhibited glucocorticoid-receptor complex activation in a dose-dependent and stereospecific manner (Figures 1 and 2). Surprisingly, H-GPT-H1 also inhibited unoccupied glucocorticoid receptor hormone-binding in a dose-dependent and stereospecific manner (Figure 3). Since the D-glycerol stereoisomer, H-GPT-H2, did not inhibit unoccupied receptor steroid-binding, the inhibition of glucocorticoid-binding observed with H-GPT-H1 is probably a specific effect, and not simply a non-specific detergent-like process. Moreover, the degree of steroid-binding inhibition by this compound was approximately the same as its ability to inhibit steroid-receptor complex activation (i.e., ~40% inhibition at ~4 mM). These results suggest that H-GPT-H1 may interact (albeit weakly) with the steroid-binding domain of the glucocorticoid receptor. Modulator and sodium molybdate are also thought to associate with the hormone-binding region of the receptor (2, 4). However, in contrast to these regulators which stabilize unoccupied glucocorticoid receptor steroid-binding, H-GPT-H1 may form a non-productive complex with the hormone-free receptor. It is important to note that H-GPT-H1 did not significantly alter the steroid-binding properties of the occupied-unactivated or occupied-activated receptors (data not shown).

Although H-GPT-H1 significantly inhibited glucocorticoid-receptor complex activation, it was clearly not as potent as sodium molybdate (Figure 1). Furthermore, this synthetic compound is about 100-1000 times less effective than modulator (14). Nevertheless, of the 26 natural and synthetic phosphoglycerides that have been tested (13, 17; and this report), H-GPT-H1 is the only one to exhibit any modulator-like effect. Thus, although modulator is not exactly mimicked by this ether phosphoglyceride, H-GPT-H1 is the first synthetic organic molecule to possess some modulator activity. Moreover, these results imply that at least part of the proposed structure for modulator (Table 1; 12, 13) is correct.

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